Developing novel therapeutic agents for *Acanthamoeba* infection and investigating the process of encystment

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Declaration

This work or any part thereof has not previously been presented in any form to the University or to any other body whether for the purposes of assessment, publication or for any other purpose (unless otherwise indicated in page 3). Save for any express acknowledgements, references and/or bibliographies cited in the work, I confirm that the intellectual content of the work is the result of my own efforts and of no other person.

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List of posters and publication related to the work presented in this thesis:

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Abstract

Acanthamoeba Keratitis (AK) is a vision-threatening disease which can lead to blinding corneal tissue infection. Many patients who have been infected with *Acanthamoeba* in their eye do not respond to the current medical treatments involving polyhexamethylene biguanide or chlorhexidine despite the *in vitro* sensitivity of *Acanthamoeba* to these drugs. There is an urgent need for new therapeutic agents to eradicate the AK infection. This study focuses on the mechanism by which *Acanthamoeba* may distinguish between trophozoite, cyst and the newly identified lifecycle known as protocyst. The current study has tested 56 novel and existing therapeutic agents for their activity against *Acanthamoeba* spp. and their toxicity against a human epithelial cell line. The results of this research have revealed several compounds of interest for further study on their potential use in the treatment of AK. These compounds included, octenidine hydrochloride, alexidine, miltefosine and quaternary ammonium (didecyldimethylammonium chloride). The anti-amoebic effect of benzalkonium chloride, povidone iodine and tetracaine are superior to the current diamidines and slightly lower to the biguanides applied in the treatment for AK. The formulation of novel amidoamine compounds including myristoleyl-amidopropyl-dimethylamine (MOPD) and palmitoleyl-amidopropyl-dimethylamine (POPD) into contact lens solutions showed complete kill at a 4.5-log reduction against trophozoites compared with myristamidopropyl dimethylamine (MAPD) as an existing compound. The combination of biguanide compounds with lipid–based carriers has improved the antimicrobial activity from 1-fold to around 7-fold against cysts of *Acanthamoeba* spp. compared with the use of biguanides alone.

The findings of encystment investigation (the transformation of trophozoites into cysts) showed that the agonists in particular the β ultra-long against indacaterol stimulated the encystment and the antagonists β_1 metoprolol blocked the formation of cysts and protocysts. Two different herbicides including 2,6-dichlorobenzonitrile (DCB) and isoxaben were tested to target the biosynthesis of cellulose in the cyst form and also to evaluate their effects on the formation of protocyst of *Acanthamoeba*. The results of this study showed that the DCB at a high concentration of 500 µM, reduced encystment to 17.7% and protocyst

production of *Acanthamoeba* at 24.6%, whereas isoxaben inhibited the transformation of trophozoites into cysts to only 45% and the percentage was decreased for protocyst formation by 37.2%. The test results for DCB and isoxaben individually at concentration of 100 uM showed 31.8% and 68.8% respectively for the conversion of trophozoites into cysts. In addition, a similar concentration of both DCB and isoxaben was evaluated for protocyst formation and the inhibition was observed at 36.9% for DCB and a much higher rate of protocysts production was recorded at 63 % for isoxaben. The combination of both isoxaben and DCB at a concentration of 100 µM caused a reduction in encystment to 49.1% and lowered the transformation of trophozoites into protocysts to 45.7%, these findings suggested that an antagonistic effect was occurred relative to the use of DCB alone. Finally, the data from LC/MS analysis for sugars suggested that the protocyst and cyst are different stages of *Acanthamoeba*, as the analysis of cyst walls indicated the presence of cellulose while the protocyst wall analysis showed the existing of cellulose and methylated sugar possibly corresponded to a methylated analogue of *N*-acetylglucosamine.

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"*Quality is never an accident; it is always the result of high intention, sincere effort, intelligent direction and skillful execution; it represents the wise choice of many alternatives*." William A. Foster.

Dedication

This thesis is dedicated to my wonderful parents, my brother, my sisters and to my nephew and niece for their love, encouragement and prayers, words cannot express my gratitude.

Abbreviations

ACAID	Anterior chamber-associated immune deviation
ACN	Acetonitrile
AgNPs	Silver nanoparticles
AK	Acanthamoeba Keratitis
ANOVA	Analysis of variance
ATCC	American Type Culture Collection
BAC	Benzalkonium chloride
cAMP	Cyclic adenosine monophosphate
CESA	Cellulose synthase
CHLX	Chlorhexidine
cm	Centimetre
CNS	Central Nervous System
COX	Cyclooxygenase
CO_2	Carbon dioxide
CSI	Cellulose synthesis inhibitors
DCB	2,6-dichlorobenzonitrile
DMAD	Dimethyl acetylenedicarboxylate
DMEM	Dulbecco’s Phosphate Buffer Saline
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DPBS	Dulbecco’s phosphate buffered saline
DPPC	Phospholipids dipalmitoylphosphatidylcholine

DTH	Delayed-type hypersensitivity
EDTA	Ethylenediaminetetraacetic acid
GAE	Granulomatous Amoebic Encephalitis
GC–MS	Gas chromatography – mass spectrometry
GPCRs	G-protein coupled receptors
g	gram
Hep2	Human epithelial cells type 2
HPLC	High Performance Liquid Chromatography
IgA	Immunoglobulin A
ISO	Organization for Standardization
Isox	Isoxaben
IVCM	*In vivo* confocal microscopy
kDa	kilodalton
KH_2PO_4	Potassium phosphate (monobasic)
LC–MS	Liquid chromatography – mass spectrometry
LC	Langerhans cells
LQ	Lipodisq®
MAPD	Myristamidopropyl Dimethylamine
MBP	Mannose-Binding Protein
MCC	Minimum Cysticidal Concentration
MCT	Minimum Cytotoxic Concentration
MeOH	Methanol
$MgCl_2$	Magnesium Chloride
MIC	Minimum Inhibitory Concentration
Min	Minutes

MOPD	Myristoleyl-amidopropyl-dimethylamine
MTAC	Minimum Trophozoite Amoebicidal Concentration
MTIC	Minimum Trophozoite Inhibitory Concentration
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
NEM	Neff's encystment medium
NSAIDs	Nonsteroidal anti-inflammatory drugs
PAM	Primary Amoebic Meningoencephalitis
PAPD	Palitamidopropyl Dimethlamine
PBS	Phosphate buffer saline
PCR	Polymerase Chain Reaction
PG	Propylene glycol
PHMB	Polyhexamethylenebiguanide
POPD	Palmitoleyl-amidopropyl-dimethylamine
PSMA	Poly(styrene-*co*-maleic acid)
qPCR	Quantitative polymerase chain reaction
RNase	Ribonuclease
rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
TEM	Transmission Electron Microscopy
v/v	volume/volume
w/v	weight/volume

x g	Gravity
°C	degree Celsius
µg	microgram
µm	micrometre
µM	micromolar
mM	Millimolar
M	Molar

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Chapter One

General Introduction

Chapter 1: Introduction

1.1 Background

Acanthamoeba is a genus of free-living amoeba which is an opportunistic protozoan pathogen in humans. There are two distinct stages of the life cycle of *Acanthamoeba:* the vegetative, replicating and feeding stage, known as a trophozoite, and the dormant stage, known as a cyst. Acanthamoeba Keratitis (AK) is a severe, sight-threatening disease which potentially causes a blinding infection of the corneal tissue. An association between AK and the wearing of contact lenses has been identified and such wearers account for up to 90% of recorded cases (Carnt *et al*., 2018). There are several reasons for the problems caused by contact lenses, including lens hygiene practices, exposure to water (Joslin *et al*., 2007), types of lens-care solutions (Verani *et al*., 2009), showering while wearing lenses (Carnt and Stapleton, 2016), the frequency with which lenses are changed (Chalmers *et al*., 2007). Non-contact lens users can also become infected with AK, but this is often related to an injury of the corneal epithelium (Garg *et al*., 2017).

Acanthamoeba reaches the eye either through contaminated water or on contact lenses and it starts to kill target cells in a contact-dependent process. There are two stages in corneal infections caused by *Acanthamoeba*. Firstly, the trophozoite adheres to the corneal epithelium mediated by a mannose binding protein (MBP) and in this primary step of adherence, mannosylated glycoproteins stimulate the liberation of a 133 kDa protein termed as the mannose-induced protease 133 (MIP-133) cysts (Hurt *et al*., 2003). Secondly, the serine protease MIP-133 causes apoptosis of the corneal epithelial cells via a cytosolic phospholipase $A_{2\alpha}$ pathway, which enables trophozoite penetration into the corneal stroma and leads to extensive damage of the collagen matrix and causes inflammation (Niederkorn *et al*., 1999). The tear fluid formed by the lacrimal system, together with constant eye lid movement, provides the key line of defence against AK infection as the human tear has IgA antibodies that inhibit the adhesion of the MBP in the trophozoite to the corneal epithelial cells (Panjwani, 2010). The mechanism of defence for IgA is the interface with (Fcα) receptors of neutrophils or macrophages and this interaction lead to stimulates the

inflammation and elimination of the *Acanthamoeba.* Supporting data for this mechanism, it has been shown that the rabbit anti-*Acanthamoeba* IgA antibody has improved the neutrophil-mediated eradicating the trophozoites *in vitro* (Said *et al*., 2004). The tear film is primarily composed of lysozyme, lactoferrin, b-lysins, immunoglobulin A (IgA) and other compounds with antimicrobial and immunological properties (Qu and Lehrer, 1998). The levels of IgA are reduced in patients with AK and the anti-amoebic IgA plays an important role in the prevention of AK infection (Walochnik *et al*., 2001). This is raised the possibility that IgA is an essential protective factor against *Acanthamoeba* infection at the corneal surface. In addition to the IgA prevention mechanism for the binding of *Acanthamoeba* trophozoites to epithelial cells, other IgA defence mechanisms have been identified including complement activation (Stewart ***et*** *al*., 1992), opsonisation (Said *et al*., 2002) and increase in neutrophil killing (Stewart *et al*., 1994).

A few of the immune mechanisms involved in corneal defence against *Acanthamoeba*. The conjunctiva is extremely vascular with lymphoid tissue and comprises mainly of IgA-generating plasma cells, T-lymphocytes, natural killer cells and macrophages, that have a high influence on the removal of *Acanthamoeba* and induce the response of humoral and T-cells (Khan, 2006). The tears comprise a complement made up of serum-borne molecules in a cascade-like manner. *Acanthamoeba* has been shown to directly activate the complement system through a substitute pathway. However, *Acanthamoeba* is resistant to complement-mediated lysis due to the expression of complement regulatory proteins, along with the decay accelerated factor (Khan, 2009b). Moreover, the regulatory complement proteins are produced in corneal cells and exist in tears, so the stimulation of the complement system has slight to no impact on AK resistance (Akpek and Gottsch, 2003). The *Acanthamoeba* genus is the aetiologic agent of a vision-threatening infectious disease that affects the human cornea known as Acanthamoeba Keratitis (AK). The increasing incidence of AK, which occurs mainly in immunocompetent individuals, is a public health problem with serious consequences for vision in view of the absence of a standard therapy and the complexity of the prognosis of the disease (Carvalho *et al*., 2009). The risk of AK infection has increased due to unlicensed treatments and the current

medical treatment involves the topical administration of polyhexamethylene biguanide (PHMB) or chlorhexidine at concentration of 0.02% (v/v) as monotherapy or in combination with diamidines (Papa *et al*., 2020).

1.2 The discovery of *Acanthamoeba*

Historically, *Acanthamoeba* research started with a work by Count Aldo Castellani in the 1930s, who detected *Acanthamoeba* as a contaminant in the culture of the yeast *Cryptococcus pararoseus* (Castellani, 1930). It is only after the work of this scientist that the amoeba was determined to have a diameter of 13.5 to 22.5 µm and the cyst form showed a double wall with an average diameter of between 9 –12 µm. Based on these characteristics, it was placed in the genus *Hartmannella* and named *Hartmannella castellanii* in recognition of Aldo Castellani (Douglas, 1930). Around 3 decades later, Clyde Culbertson and colleagues discovered a contamination in a monkey kidney cell line whilst developing a polio vaccine (Culbertson *et al.*, 1959, Culbertson *et al.*, 1958). The existence of plaques on these cells led the researchers to believe that the contaminant was viral. However, when mice and monkeys were injected with these contaminates, they quickly died from fatal meningoencephalitis (Culbertson, 1971). Subsequently, the causative agent and contaminant was identified as *Acanthamoeba* and this was the first time that the free-living amoeba *Acanthamoeba* had been shown to be pathogenic. The strain was later termed *Acanthamoeba culbertsoni* (Singh and Das, 1970). The finding that *Acanthamoeba* spp. can cause disease in animals suggested that it could also be pathogenic to humans. The first recorded case of human infection related to *Acanthamoeba* spp. was in a patient with Hodgkin's disease (HK) who developed granulomatous amoebic encephalitis (GAE) (Jager and Stamm, 1972). HK is a kind of lymphoma, which is a blood cancer that begins in the lymphatic system (Banerjee, 2011). The potential cause of HK patients being infected with GAE is related to their poor immune system (Bentivoglio *et al*., 2014).

1.3 Classification of *Acanthamoeba*

The genus *Acanthamoeba* was placed in the order of Amoebida, but it is now classified within the order of Centramoebida to differentiate it from Theacamoebida, the suborder Acanthapodina, and the family Acanthamoebidae. It is a free-living amoebae (FLA) found in soil, water and the environment. In

1975, the family Acanthamoebidae was first proposed and included the spiny or thread-like pseudopod-forming, slug-like amoebas that can form smooth, spherical cysts (Sawyer and Griffin, 1975). It was established that the *Acanthamoeba* species can be divided into three morphological groups, I, II and III, based on the size and shape of the cysts (Pussard and Pons, 1977). Group I of the *Acanthamoeba* species is designated on the basis of it having a large cyst. Group II is characterized as having a wrinkled ectocyst and an endocyst. Group III has a smooth endocyst and a rounded ectocyst (Pussard and Pons, 1977). However, this method of classification according to the morphology of the cyst has since been found to be uncertain, due to the fact that culture conditions can affect the morphology of the cyst (Stratford and Griffiths, 1978).

Genetic typing based on 18S ribosomal RNA genes has been used to classify the *Acanthamoeba* into sequence groups from T1-T12 regarding human diseases associated. 18S ribosomal RNA is the structural RNA of a small component of the eukaryotic cytoplasmic ribosome, such that Group I includes sequence types T7, T8, T9, Group II includes sequence types T3, T4, T11, and Group III includes sequence types T1, T2, T5, T6, T10, and T12. So far, the genus *Acanthamoeba* has been classified into 15 different genotypes from T1 to T15, based on rRNA sequences (Schuster and Visvesvara, 2004a). On the other hand, around 20 genotypes have been identified (T1–T20) according to the analysis of the ribosomal DNA (rDNA) gene of *Acanthamoeba* (Corsaro *et al.*, 2015). One year later, another genetic study identified two more genotypes induced T21 and T22 of *Acanthamoeba* through an analysis rDNA sequences (Tice *et al*., 2016). Prior investigations in the UK and Iran have looked at the genotype of AK isolates and it has been found that the highest numbers come from the T4 group (70.8%), whereas smaller numbers are from T2 (12.5%), T3 (12.5%), and T11 (4.1%) (Maghsood *et al*., 2005). In addition, the same authors have proposed that the T2 genotype should be sub-divided into two groups, T2a and T2b, because of a sequence variation of 4.9% between these two groups (Maghsood *et al*., 2005).

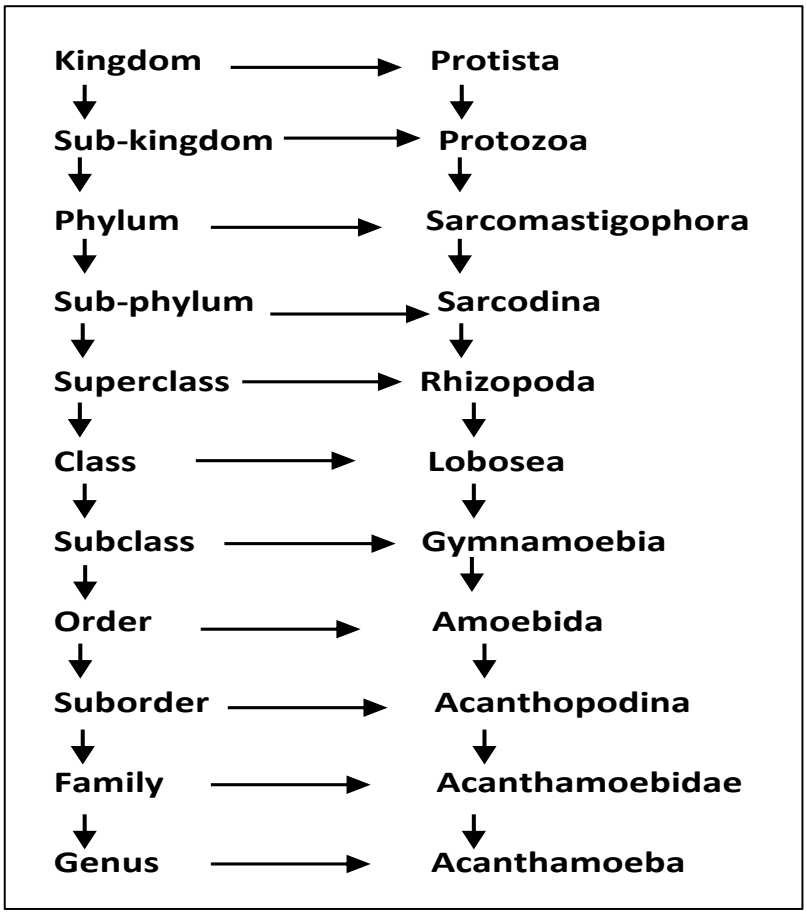

Figure1-1:Classification scheme of Protist, based largely on morphological characteristics. Modified from (Marciano-Cabral and Cabral, 2003).

Most of the studies in the scientific literature have suggested that there are only two distinct stages in the lifecycle of *Acanthamoeba,* namely the trophozoite, which is the motile form and the double layered, dormant, mature cyst form (Siddiqui *et al*., 2012, Marciano-Cabral and Cabral, 2003). The cyst stage of the organism is only produced under adverse conditions while the trophozoite stage form when the conditions become favourable. However, there is another possible stage of *Acanthamoeba* known as a pseudocyst/protocyst, The protocysts are extremely resistant to the detergent lysis and they took less than 1 hour to form, in contrast with cysts which take more than 24 hours to form. Moreover, protocysts could not mature into cysts unless they were first transformed into trophozoites as clarified in Figure 1.2 (Kilvington *et al*., 2008).

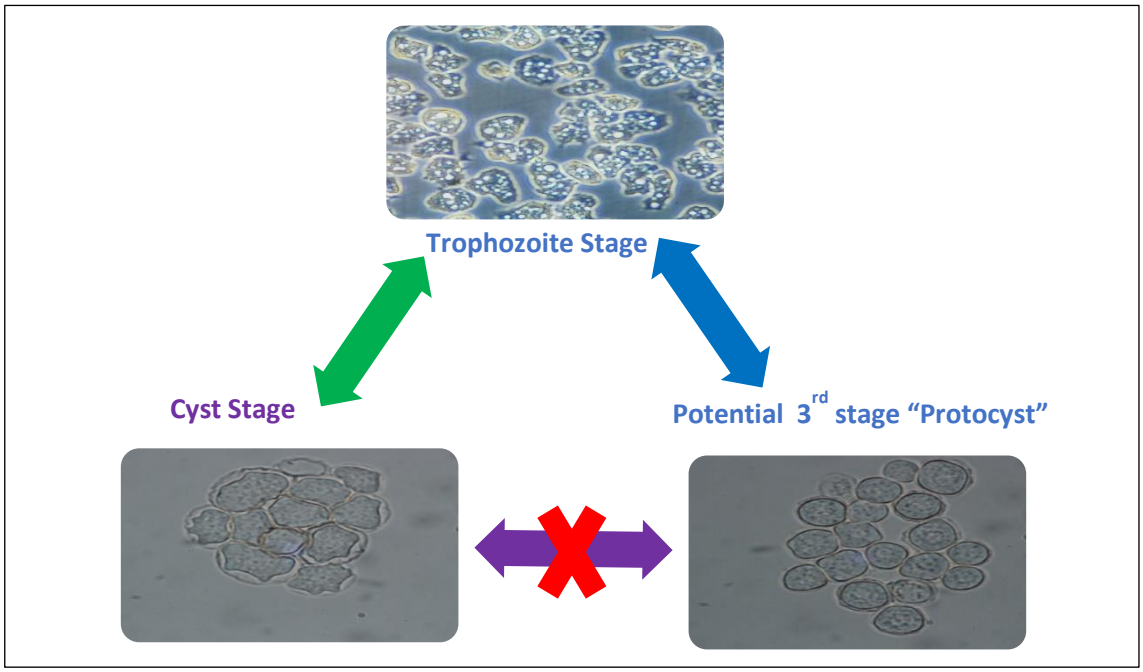

Figure1-2:Showing the life cycle of *Acanthamoeba*

1.4.1 The trophozoite stage of *Acanthamoeba*

The term 'acanth' (a Greek word meaning "thorns") was added to amoeba because of the existence of spine-like structures on the surface of trophozoites. These are now known as acanthopodia (as can be seen in Figure 1.3) and they are very important for adhesion to surfaces on hosts and for movement (Bowers and and Korn, 1973). The trophozoite is the vegetative stage of the *Acanthamoeba* in which the amoeba reproduces by binary fission (Martinez, 1996). Under the inverted light microscopy, the trophozoite appears as an irregular, amoeboid-shaped organism and ranges in size between 20 and 45 µm. The trophozoites of *Acanthamoeba* possess a single nucleus with a nucleolus, abundant cytoplasm with multiple elongated mitochondria, lysosomes, endoplasmic reticulum, ribosomes, Golgi apparatus and vacuoles (Bowers and Korn, 1968, Martinez and Visvesvara, 1997). The main food source for *Acanthamoeba* is bacteria (Weekers *et al*., 1993). *Acanthamoeba* feed through the creation of a temporary structure named a food cup. The food cup is part of the cell wall and is expected out to grab bacteria, yeast and cellular debris (Pettit *et al*., 1996).

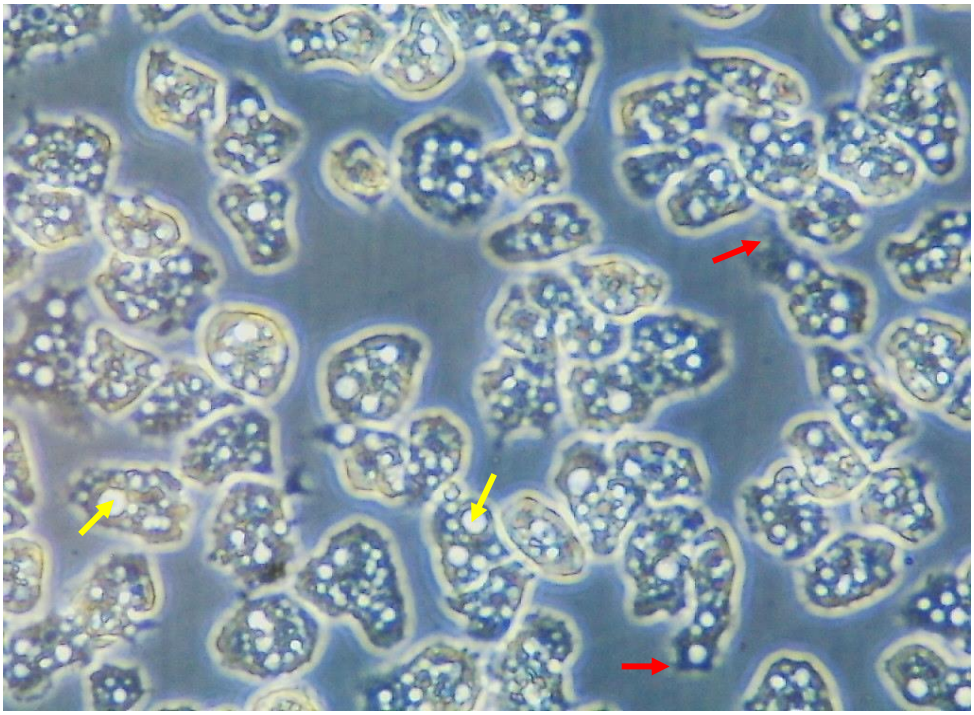

Figure1-3: The trophozoite stage of *Acanthamoeba castellanii* (ATCC 50370) with clear contractile vacuole (yellow arrows) and acanthopodia (red arrows). Observed with an inverted light microscopy at magnification x400.

Acanthamoeba uses two distinct pathways to take up food: a nonspecific pathway called pinocytosis for the uptake of soluble material and another, highly-specific, receptor-mediated pathway called phagocytosis (Chambers and Thompson, 1976). The pathogenic strains of *Acanthamoeba* have been shown to bind to target cells by using a greater number of acanthapodia structures. It has also been pointed out that the phagocytosis process in *Acanthamoeba* occurs through the formation of amoebastome, which is a characteristic of the *Acanthamoeba* phagocyte (Khan, 2001). The trophozoite is characterized by the existence of a large contractile vacuole, as illustrated in Figure 1.3 above, which periodically expels and then refills with water for the cell, and possibly excretes waste (Bowers and Korn, 1973). The force required to contract the vacuole is created by myosin which is used to regulate the water content of the cell. The contractile vacuole absorbs water by osmosis from the cytoplasm and moves to the surface of the cell when full and undergoes exocytosis (Doberstein *et al*., 1993).

1.4.2 The cyst stage of *Acanthamoeba*

The cyst is the dormant stage of *Acanthamoeba* and forms when the organism encounters unfavourable conditions, such as hyper- osmolarity (Cordingley *et al*., 1996), heat cold desiccation and cysts can lay dormant for several years until food becomes available (Heaselgrave and Kilvington, 2016, Neff *et al*., 1964). The cyst is smaller than the trophozoite at about 12-20 µm in diameter, depending on the species, and under transmission electron microscopy, a thick cyst wall can be observed consisting of two distinct layers: the endocyst and the ectocyst. In the cytoplasm, the nucleus appears as a rounded structure which is black in colour with a nucleolus and the mitochondria is also visible as shown in Figure 1.4. A & B. Furthermore, the most common holes to appear on the surface of the cyst wall are called ostioles. The excystment process occurs through these ostioles after digestion of the opercula covering the ostioles and they are also used to monitor environmental changes (Chavez-Munguia *et al*., 2005).

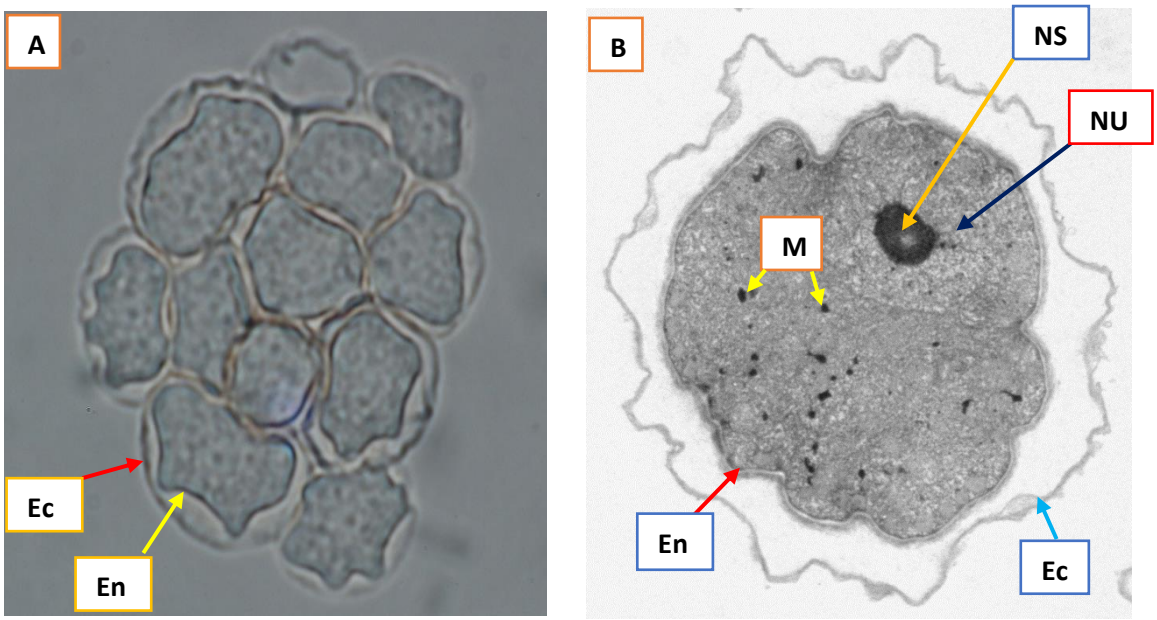

Figure1-4:Morphology of *Acanthamoeba castellanii* (ATCC 50370) mature cysts. A) Non-Nutrient Agar (NNA) mature cyst with a double-walled viewed under phase contrast light microscopy at magnification x1000. (B) Transmission electron microscopy showing the double-walled of Neff's mature cyst with ectocyst (Ec), endocysts (En), nucleus (NS), nucleolus (NU) and mitochondria (M), the scale bar is 0.2 µm.

The ectocyst has an irregular surface and by utilising quick-freeze, fracture/deep etching with TEM imaging, it has been shown that the endocyst is thinner and more fibrillar than the ectocyst (Lemgruber *et al*., 2010). The reasons for encystment have not been fully clarified. However, a cyst-specific proteins 21 kDa and 70 KDa in size, have been identified which are not found in the trophozoite stage of *Acanthamoeba*. This specific proteins are purified from guanidine-HCl extract in the cyst wall and has been shown to be hydrophilic and the expression of a cyst-specific protein is restricted to the early stages of encystment (Hirukawa *et al*., 1998, Rubin *et al*., 1976), suggesting that the biosynthesis of cyst-specific proteins 21 and 70 KDa are regulated at the mRNA level. *Acanthamoeba* cysts are extremely resistant to harsh environmental conditions and chemical disinfection due of the presence of the thick cyst wall. Previously, a soft multipurpose solution of MeniCare has been tested against cysts and it showed more than a 3-log reduction against the cysts of *Acanthamoeba* after 6 hours of exposure (Heaselgrave *et al*., 2010). Also, a hydrogen peroxide solution at a concentration of 7.5% with 30 minutes exposure has been shown to have only limited activity against different strains of *Acanthamoeba* isolated from river water and from a hospital water network (Coulon *et al*., 2010).

It has been previously demonstrated that the effect of different agents on *Acanthamoeba* cysts is dependent on the production method of the cysts and the age of the tested strain. PHMB at a concentration of 3 µg/mL achieved a 3 to 4 log kill against Neff's and non-nutrient agar (NNA) cysts of *Acanthamoeba polyphaga* (Ros-91 strain) after 6 hours of exposure, while the cysts from another tested strain, Ros-02 of *A. polyphaga,* were found to be more resistant to PHMB at the same concentration and exposure time, giving a 0.3 log kill for Neff's cysts and 1.2 log kill against NNA cysts (Hughes *et al*., 2003b). Myristamidopropyl dimethylamine (MAPD) has been tested at a concentration of 20 µg/mL and exhibited activity over 6 hours between a 2.8 and 4 log reduction against Neff's cysts and NNA cysts of *A. polyphaga* Ros-91 strain. The activity of MAPD improved against NNA and Neff's cyst of *A. polyphaga* Ros-02 strain, ranging from a 2.7 log kill to a 4.5 log after 6 hours (Hughes *et al*., 2003b).

1.4.3 The discovery of *Acanthamoeba* pseudocyst/protocyst

Latest outbreak of AK among contact lens users in the United States resulted to the withdrawal of the Complete Moisture-Plus® contact-lens solution from the market for concerns regarding its contamination with *Acanthamoeba* (Verani *et al.*, 2009). Attempts were made to develop new products for the disinfection of contact lenses (Kilvington *et al*., 2008). During the development of such products, an unusual, immature protocyst was observed with a single cell wall, see Figure 1.5 (Kilvington *et al*., 2008). Earlier research into amoeba found that they contain a range of carbohydrate polymers. For example, *Acanthamoeba* and Balamuthia cysts contain cellulose (a carbohydrate polymer) (Lemgruber *et al*., 2010), whilst *Entamoeba* has been found to contain chitin and *Giardia* has *N*-acetylglucosamine (Samuelson and Robbins, 2011). There is a clear difference in morphology between mature cysts and protocysts of *Acanthamoeba*: the protocyst consists of a single cell wall which provides an effective barrier against drug treatments (Kilvington *et al*., 2008).

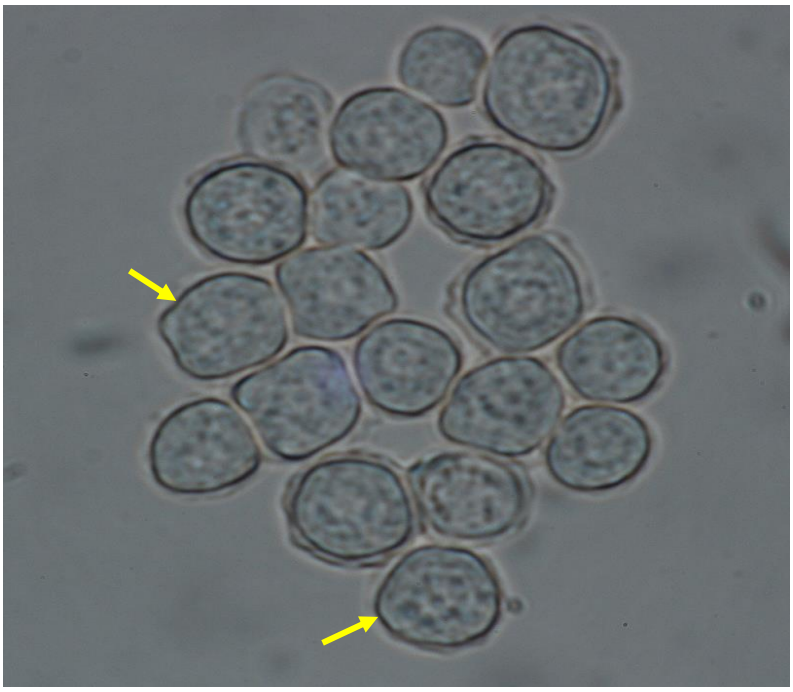

Figure1-5:Shows the immature protocyst with a single cell wall (yellow arrow) of *Acanthamoeba castellanii* (ATCC 50370), viewed under light phase contrast microscopy at magnification x1000.

1.5 Distribution of *Acanthamoeba*

Acanthamoeba has been isolated from a wide variety of environmental sources and represents a sanitary risk for public health, particularly for contact lens users and immunocompromised patients (Rezaeian *et al*., 2008). It has been isolated from bathroom and kitchen sinks (Carnt *et al.*, 2020a) and has also been found in a variety of sources including soil, different types of water, dust, hospital units and ventilation areas (Schuster and Visvesvara, 2004a), air samples, laboratory eyewash stations and bacteria or cell culture media (Astorga *et al*., 2011). Furthermore, *Acanthamoeba* has been isolated from seawater, chlorinated swimming pools, domestic tap water, bottled water, dental treatment units (De Jonckheere, 1991). The distribution of *Acanthamoeba* is not restricted only to water environments, as *Acanthamoeba* was found to exist in air samples from urban sites in a city in Mexico, with approximately 40% posing a potential pathogenic health risk (Rodriguez-Zaragoza and Magana-Becerra, 1997). *Acanthamoeba* has been isolated from the tap water of the homes over the seasons, and the findings showed that 29.6% the total of isolates during the summer and the percentage slightly decreased to 27.9% of the total isolates over the winter (Carnt *et al*., 2020b).

1.6 Adrenergic receptors and *Acanthamoeba* encystment

The adrenergic receptors are broadly classified into two categories included α and β types and they are further subdivided into 1 and 2 subtypes with their agonists and antagonists as presented in Table 1.1. There are numerous research studies concerning the ways in which *Acanthamoeba* senses and responds to environmental changes and also the signalling pathways involved in the encystment of *Acanthamoeba*. It was showed that the encystment process of *Acanthamoeba* can be mediated through catecholamines (Heaselgrave and Kilvington, 2016). The authors initially tested different concentrations of adrenoceptor agonists including epinephrine (5 mM), isoproterenol (1mM) and the selective β_1 adrenoceptor agonist dobutamine (100 µM) which were found to produce levels of encystment of 93.2%, 92% and 92.4% respectively. When the selective β_2 agonist salbutamol was tested at a lower concentration of 10 µM, the encystment level decreased to 39%. However, the authors concluded that

salbutamol caused massive lysis of *Acanthamoeba* trophozoites at higher concentrations.

Testing of the α/β nonspecific agonist epinephrine on its own at a concentration of 5 mM showed a higher level of encystment at 93.2%. In contrast, the encystment process was blocked when the selective β_1 antagonist metoprolol was combined with epinephrine at a concentration of 500 µM, giving only 18.8% encystment. The same study investigated the effect of different antagonists, including the non-selective β alprenolol, the non-selective α phentolamine and β_2 propranolol in the presence of 500 µM of epinephrine. The outcomes showed that α phentolamine had no effect on the encystment of *Acanthamoeba*, whereas β, β_2 antagonists alprenolol and propranolol reduced the encystment level to around 30% (Heaselgrave and Kilvington, 2016). An earlier study by Coppi *et al*. (2002) showed that the trophozoites of *Entamoeba* release and respond to catecholamine when it triggers encystment. Using agonists and antagonists to induce encystment in *Entamoeba* was found to be in line with the existence of a receptor with the ligand specificities of the β_1 adrenoreceptor in mammalian cells. However, the α adrenoreceptor ligands were observed to have no effect on the encystment of *Entamoeba* (Coppi *et al*., 2002).

Table 1-1: Types of adrenergic receptors and their agonists and antagonists

Receptor Type	Agonists	Reference	Antagonists	Reference
α_1	Methoxamine Midodrine Metaraminol Phenylephrine Amidephrine Epinephrine	(Kalis, 2003) (Izcovich *et al*., 2014) (Block *et al*., 1988) (Declerck *et al*., 1990) (MacLean *et al*., 1989)	Terazosin Doxazosin Silodosin Alfuzosin Tamsulosin	(Oh *et al*., 2007) (Lepor, 2007) (Rossi and Roumeguere, 2010)
α_2	Guanfacine Xylazine Norepinephrine Amitraz Medetomidine	(Sagvolden, 2006) (Atalik *et al*., 2000) (Lemke, 2004) (Hsu and Lu, 1984) (Sinclair, 2003)	Atipamezole Idazoxan Mirtazapine Yohimbine	(Baker *et al*., 2011) (Jackson *et al*., 1991) (Alam *et al.*, 2013) (Ostojic, 2006)
β_1	Dobutamine Isoprenaline Norepinephrine Epinephrine β ultra-long agonist Indacaterol	(Vallet *et al*., 1991) (Lieberman and Marks, 2009) (Cazzola *et al*., 2013)	Metoprolol Atenolol Betaxolol Nebivolol	(Ros *et al.*, 1978) (Chidlow *et al*., 2000) (Zeitz *et al.*, 2008) (Flower et al., 2016)
β_2	Formoterol Isoprenaline Salbutamol Clenbuterol Salmeterol	(Berger, 2006) (Ullmann *et al*., 2015) (Pasotti *et al*., 1979) (Perez, 2006)	Butaxamine ICI-118,551 Levobunolol Propranolol	(Wandel *et al*., 1986) (Merté and Merkle, 1980) (Thal *et al*., 2012)

1.7 Anatomy of the human eye

The human eye is composed of an anterior (front) segment and a posterior (back) segment. The front section consists of the cornea, iris and lens as illustrated in Figure 1.6.A (Oyster, 1999). The cornea is made up of six layers: the epithelium, Bowman's layer, the stroma, Dua's layer, Descemet's membrane and the endothelium (Sridhar, 2018), as can be seen in Figure 1.6.B.

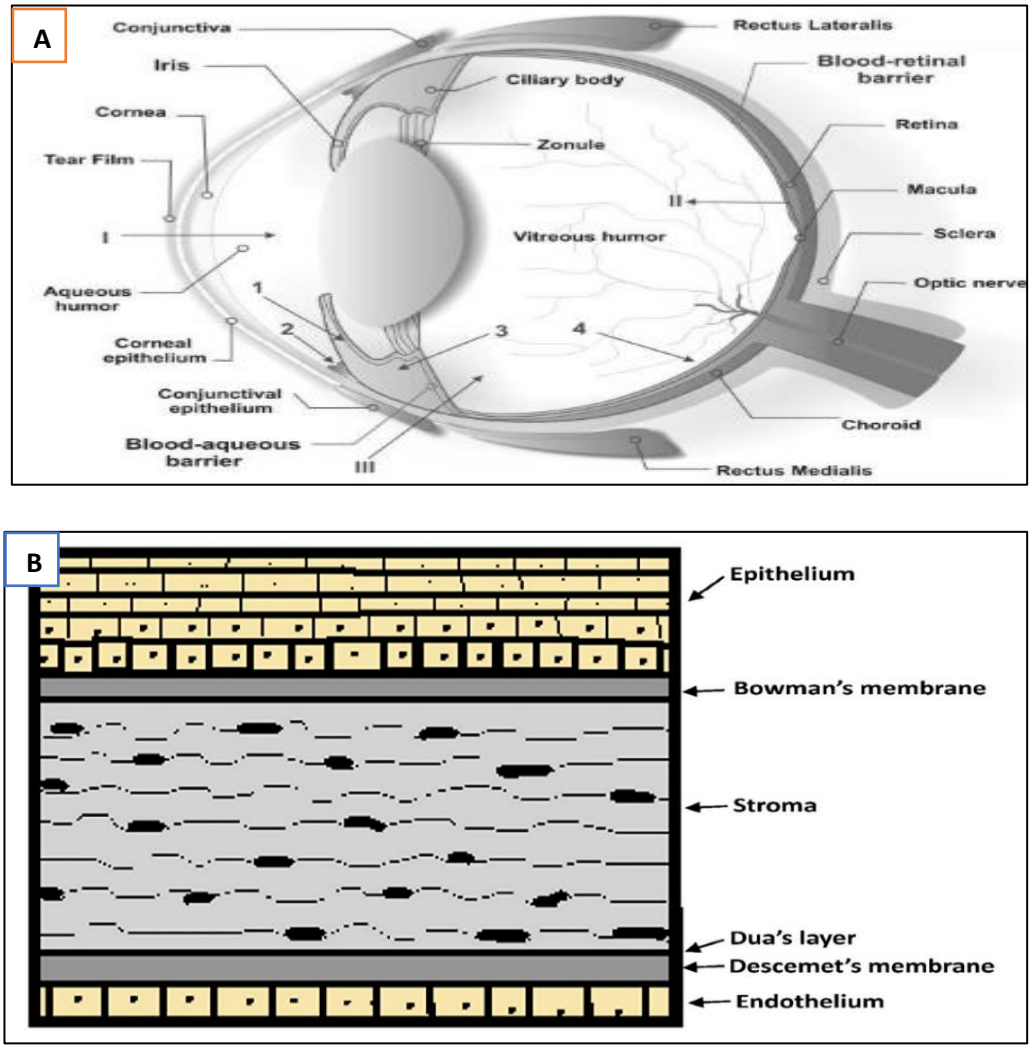

Figure1-6:The anatomy and the main components of the human eye. (A) The anterior section consists mainly of the cornea, iris, pupil, conjunctiva and the lens, the posterior section comprises of sclera, choroid and the retina. The source for image is Barar *et al*. (2009). (B) The cornea is composed of six layers. Image taken from (Varela-Fernández *et al*., 2020).

The epithelium is composed of 5–7 layers of epithelial cells connected with tight junctions. The epithelium layer is the major barrier which limits the rate of corneal penetration for most topical drugs, although lipophilic compounds have a tendency to penetrate the epithelium layer more easily than hydrophilic compounds (Prausnitz and Noonan, 1998). The reason for this difference is related to the corneal epithelium as it facilitates the absorption of lipophilic compounds (Davies *et al*., 2013). The stroma layer is located between the epithelium and the endothelium and accounts for about 90% of corneal thickness (Meek and Knupp, 2015). It predominantly consists of hydrated collagen fibrils that form a barrier which diffuses the penetration of lipophilic drugs. The stroma also aids in repairing damaged cells (Khan and Siddiqui, 2009). The corneal stroma is not structurally homogeneous throughout its depth and there is a difference between the anterior and posterior stroma. For example, the posterior stroma is more hydrated and susceptible to swelling than the anterior stroma (Freund *et al*., 1995).

1.8 Pathogenicity of *Acanthamoeba*

Pathogenic mechanisms were found most often in the strain of *Acanthamoeba* associated with AK rather than in those associated with encephalitis of the brain. The pathogenesis of infection and the biochemical determinants of virulence are poorly understood. However, the growth rate, adherence properties, cytolytic products formed by amebae, and immune evasion mechanisms seem to constitute the key factors in the pathogenicity of *Acanthamoeba* (Marciano-Cabral and Cabral, 2003). The virulence factors in *Acanthamoeba* may be related to the distinct physiological characteristics of the strain rather than depending on suitable environmental conditions (Walochnik *et al*., 2000). It has been shown that the glycolipids of corneal epithelium react with *Acanthamoeba* and could also play an important role in the pathogenesis of AK. This occurs through mediation of the adherence of the amebae to the cornea (Panjwani *et al*., 1992). The adhesion molecule, which is distinct from the 136-kDa mannose-binding protein (MBP), plays a key role in the attachment of amebae to the host cells (Kennett *et al.*, 1999). *In vitro* studies have used human epithelial cells, stromal keratocytes, and stromal cell homogenates as models of AK. Based on such studies, it is thought that damage to cells and tissue occurs through phagocytic processes

and via cytotoxic substances which are released by the *Acanthamoeba* (Stopak *et al*., 1991).

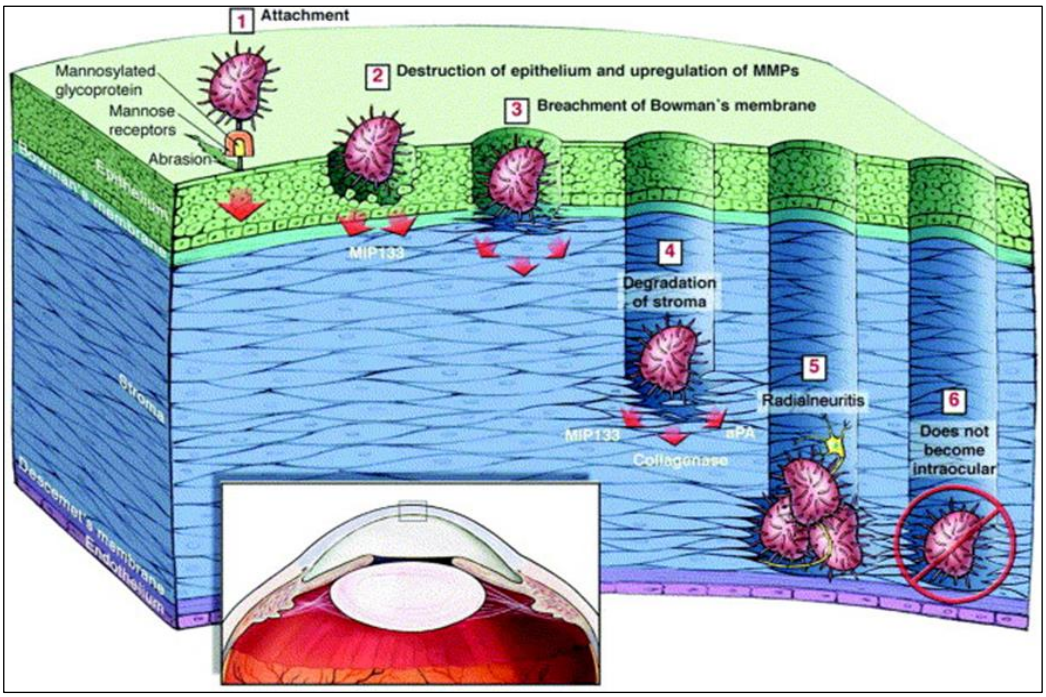

Figure1-7: Showing the pathogenic steps of AK. Step (1) The trophozoites of *Acanthamoeba* attach to mannosylated glycoproteins, which are involved in the regulation on corneal epithelium in response to corneal abrasion. Step (2) Mannose exposure induces trophozoites of *Acanthamoeba* to release MIP133. Step (3) Trophozoites destroy Bowman's membrane and penetrate the collagenous stroma. Step (4) Trophozoites continue producing a number of proteases that contribute to the dissolution of the corneal stroma. Step (5) Trophozoites frequently clumps around corneal nerves, generating radial keratoneuritis and severe pain. Final step (6) AK very seldom progresses beyond the corneal endothelium to cause intraocular diseases. The source of image is Clarke and Niederkorn (2006).

The pathogenesis of *Acanthamoeba* is extremely complex and involves numerous factors working together to produce the disease. The sequence of stages of infection for AK starts with penetration of the surface of the epithelium and keratocyte depletion by *Acanthamoeba.* Following this, the amoeba creates stromal necrosis and induces an intense inflammatory response (Vemuganti *et al*., 2004, Garner, 1993). Several studies have revealed that the majority of strains causing AK belong to sequence type T4 (Schroeder *et al*., 2001, Stothard

et al., 1998, Walochnik *et al*., 2000). It is still unclear why the *Acanthamoeba* T4 genotype is the most abundant in human disease. However, it has been proposed that the main reasons for *Acanthamoeba* T4 isolates being linked with human infections are their greater virulence factors which improve their transmissibility and their reduced susceptibility to chemotherapeutic agents (Maghsood *et al*., 2005). Many species of the genus *Acanthamoeba* have been linked with AK infection. According to the classification scheme provided in Table 1.2, most human infections are associated with isolates of the T4 genotype. For example, over 90% of keratitis disease has been associated with the T4 genotype. Similarly, T4 has been the main genotype linked with the disease encephalitis (Siddiqui *et al.*, 2012).

Table 1-2: Classification of *Acanthamoeba* genotypes based on the rRNA sequences and their links with human diseases. The table is redrawn from (Khan, 2006).

Acanthamoeba genotypes	Human disease association
T1	*Acanthamoeba* encephalitis
T2a	*Acanthamoeba* Keratitis & encephalitis
T2b	No disease association
T3	*Acanthamoeba* Keratitis
T4	*Acanthamoeba* Keratitis & encephalitis
T5	*Acanthamoeba* Keratitis
T6	*Acanthamoeba* Keratitis
T7	No disease association
T8	No disease association
T9	No disease association
T10	*Acanthamoeba* encephalitis
T11	*Acanthamoeba* Keratitis
T12	*Acanthamoeba* encephalitis
T13	No disease association
T14	No disease association
T15	No disease association

1.9 Granulomatous amoebic encephalitis (GAE)

Acanthamoeba is one of the causative agents of granulomatous amoebic encephalitis (GAE) which is a chronic disease of the central nervous system (CNS). GAE is an uncommon and often fatal infection, found most commonly in immunocompromised or severely debilitated individuals and this is the possible reason why a patient infected with Acanthamoeba Keratitis does not get GAE. The clinical symptoms for GAE include headache, fever, behavioural changes, aphasia, ataxia, vomiting, and seizures (Harwood *et al.*, 1988, Kernohan *et al*., 1960). GAE was first observed in 1972 (Jager and Stamm, 1972), and the term granulomatous amoebic encephalitis (GAE) was proposed 8 years later to describe the histopathological features of the disease (Martínez *et al*., 1980).

GAE is uncommon, subacute, necrotising infection most commonly caused by *A. castellanii*, *A. culbertsoni*, or *A. polyphaga* and the related amoeba *Balamuthia mandrillaris* (Jayasekera *et al.*, 2004). Other factors that can cause GAE include chemotherapy, steroid treatment, alcoholism, radiation therapy, systemic lupus, acquired immune deficiency syndrome (AIDS), and haematological disorders (Martinez and Visvesvara, 1997). GAE is different to primary amoebic meningoencephalitis (PAM), which is caused by an amoeba called *Naegleria fowleria*. This amoeba enters the brain in two stages. Firstly, the aspiration of contaminated water into the nasal cavity causes the entry of the amebae. Secondly, the amoeba penetrates the nasal epithelium and then moves up the olfactory nerve tracts to the brain (Schuster and Visvesvara, 2004a). A longer incubation period, ranging from weeks to months, has been recorded with GAE infection which differs from *Naegleria* infection (Schuster and Visvesvara, 2004a). The primary routes for *Acanthamoeba* to enter the body are through broken skin, the lower respiratory tract or the genito-urinary tract and it then moves around the body in the blood stream before reaching the CNS through hematogenous spread as shown in Figure 1.8 (Martinez and Visvesvara, 1997).

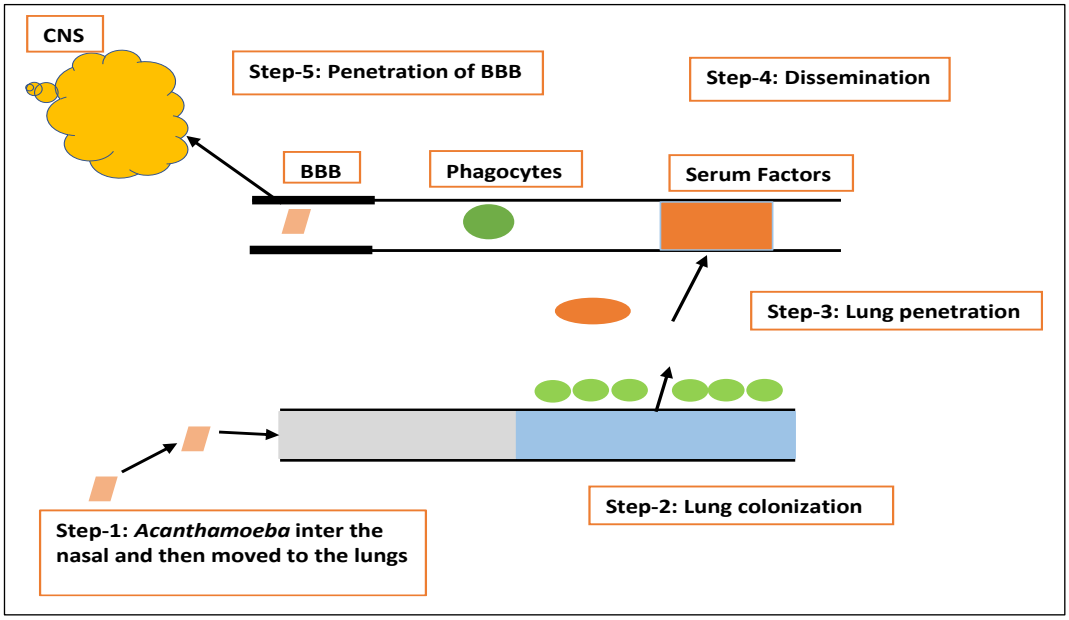

Figure1-8: Diagram presenting the *Acanthamoeba* granulomatous encephalitis infection, *Acanthamoeba* may use several routes to enter the human body, including the nasal route into the lungs followed by an invasion of the bloodstream leading to haematogenous spread. The entry of the amoebae into the CNS most likely occurs at the site of the blood-brain barrier (BBB). The olfactory nerve may provide an alternative route of entry into the central nervous system. The diagram is modified from Khan (2005).

There is no evidence exists of the mechanisms used by *Acanthamoeba* to cross the blood barrier in humans. However, *Acanthamoeba* secretes a serine protease enzyme which leads to an increase of 45% in the permeability of the blood barrier and results in the breakdown of microvascular endothelial cells (Alsam *et al.*, 2005). It has been reported that the non-pathogenic *Acanthamoeba* lacks serine protease and is unable to exert a cytopathic effect on *in vitro* cell cultures (Khan *et al.*, 2000).

1.10 Acanthamoeba Keratitis (AK)

AK infections are mainly associated with wearing of contact lenses. Patients infected with AK may suffering from a range of symptoms included pain with photophobia, epithelial defects and ring-like stromal infiltrates as shown in Figure 1.9 below (Illingworth *et al*., 1995, Lindsay *et al*., 2007, Graff *et al*., 2006). The appearance of a ring infiltrate inside the cornea, thought to be made up of infiltrating inflammatory cells such as macrophages/neutrophils in response to the

Acanthamoeba infection (Marciano-Cabral and Cabral, 2003). AK is associated with pain due to the fact that *Acanthamoeba* has a specific affinity to the nerve tissue and thus induces inflammation of the nerves in the cornea and hence pain. Several studies in the medical literature have revealed that if AK is not identified at an early stage and not treated adequately and aggressively, it may lead to loss of vision (Marciano-Cabral and Cabral, 2003, da Rocha-Azevedo *et al*., 2009, Visvesvara *et al*., 2007, Visvesvara, 2010). *Acanthamoeba* strains isolated from patients with severe AK secrete proteolytic enzymes of serine-like proteases. When the trophozoites reaches the corneal epithelium, two enzymes, gelatinases and collagenases, could be implicated in the activation of pathogenic cascades and subsequent digestion of the collagenous corneal stroma (de Souza Carvalho *et al*., 2011). AK patients suffer severe eye pain which is thought to be associated with radial keratoneuritis and the trophozoites found along the corneal nerves lead to a thickening and distortion of these nerves (Yu *et al*., 2004). Subsequent stages of the infection can result in epithelial denudation, stromal necrosis (Rocha-Azevedo *et al*., 2009), nerve oedema and retinal detachment and if misdiagnosed or if a delay in treatment occurs, the infection will almost certainly result in blindness as the necrosis spreads inwards (Niederkorn *et al*., 1999)

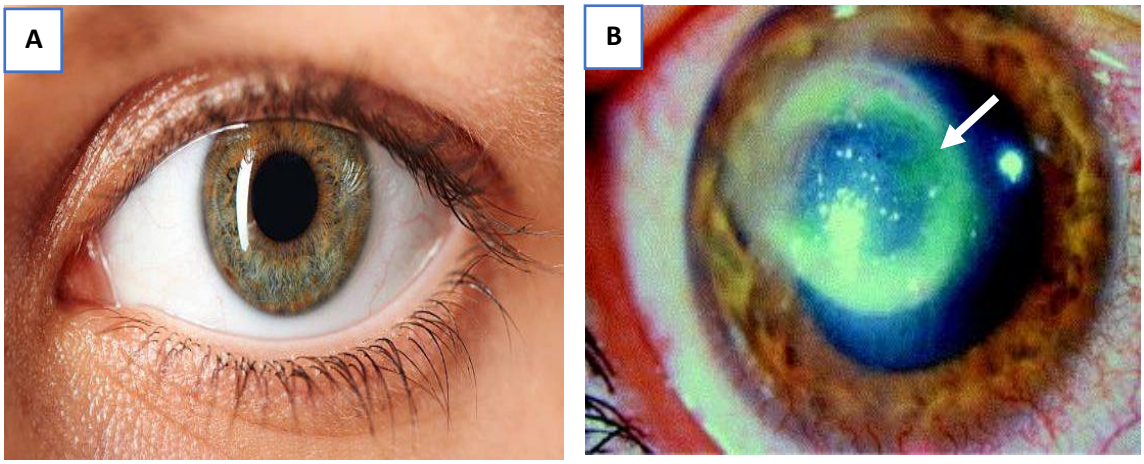

Figure1-9: Clinical characteristics of Acanthamoeba Keratitis. A) Normal human eye, the image taken from(Online Science Notes, 2018). B) The white ring infiltrate (white arrow) can be seen within the central section of the cornea. The source of image is Clarke and Niederkorn (2006).

1.10.1 *Acanthamoeba* Keratitis and the immune response

In spite of the widespread prevalence of *Acanthamoeba* in a variety of environments, the rate of infection related to AK persisted very low. This is based on the fact that *Acanthamoeba* are opportunistic pathogens, and their ability to produce diseases relies on the sensitivity of the host, environmental conditions and their own virulence (Khan, 2006). The antibodies in the serum do not protect against eye infection and inoculation of *Acanthamoeba* into the eye does not generate an adaptive immune response. The immune privilege of the cornea is partly due to the extraordinary lack of resident antigen cells in the central of the corneal epithelium. The peripheral antigen cells, called Langerhans cells (LC), can be provoked to move to the central of the cornea. The existence of LC in the cornea prior to the *Acanthamoeba* trophozoites infection has robbed the cornea of its immune privilege and has led to the production of delayed-type hypersensitivity (DTH) or serum IgG antibody responses (Klink *et al*., 1997). The failure of corneal infections with *Acanthamoeba* trophozoites to stimulate an adaptive immune response is correlated with the immune privileged nature of the eye (Klink *et al*., 1997). The trophozoites recognised by Toll-like receptor-4 (TLR4), which begins the nuclear factor kappa B (NF-kB) and the MAPK/ERK pathways, and this contributes to the production of pro-inflammatory and chemotactic mediators such as interleukin 8 (IL-8) and tumor necrosis factor alpha (TNF-α), and interferon- β (IFN-β) and these mediators have no direct anti-amoebic activity on killing trophozoites (Ren *et al*., 2010, Alizadeh *et al*., 2014).

Eye-derived immunological tolerance is termed anterior chamber-associated immune deviation (ACAID) and refers to a phenomenon in which antigen-specific systemic immunological tolerance is induced by an antigen that has been introduced to the anterior chamber of the eyes (Streilein, 2003). ACAID is an adaptive immune response and once the trophozoites of *Acanthamoeba* are attached to the corneal epithelium, ACAID does not produce a response to protect against AK infection. In the corneal transplant, the ACAID had a positive impact on the survival of the corneal allograft in the mice model and blocking of the ACAID by the destruction of the camero-splenic axis in mice resulted in an increase in the risk of corneal allotransplant refusal (Niederkorn and Mellon, 1996). The ACAID mediates the corneal transplants by producing antigen-

specific downregulation of T-helper 1 immune responses (Niederkorn, 2006). It has been revealed that components of the adaptive immune response include complement fixing antibodies, which are unable to kill the trophozoites and cysts of *Acanthamoeba* (Clarke and Niederkorn, 2006). On the other hand, the use of killed trophozoites in mucosal immunisation trigger the production of IgA antibodies secreted in tears that prevent the adhesion of trophozoites to the epithelium layer. However, if immunisation is performed after trophozoites have penetrated the cornea, no activity against AK infection occurs (Clarke and Niederkorn, 2006).

Macrophages and neutrophils are an innate immune response found in the tissues surrounding amoebic trophozoites and cysts, with macrophages capable of phagocytosis and killing *Acanthamoeba* cysts and trophozoites by inducing an inflammatory response, in particular secretion of macrophage inflammatory protein 2 (MIP-2) and leading to inflammation (Hurt *et al*., 2003, Marciano-Cabral and Toney, 1998). Treatment with anti-*Acanthamoeba* antibodies increases the amoebicidal activity of activated macrophages, most likely by increasing immune recognition (Marciano-Cabral and Toney, 1998). Inhibition of the conjunctival macrophage population was found to lead to an exacerbation in the severity and duration of AK infection in Chinese hamster models (van Klink *et al*., 1996). These findings highlight the role of macrophages in the defence against *Acanthamoeba*. However, macrophages are limited in their ability to kill trophozoites and this is likely due to the interactions of macrophage with trophozoites are not unidirectional. Furthermore, neutrophils can eradicate *Acanthamoeba* cysts and trophozoites by a myeloperoxidase-dependent mechanism and it has been shown that neutrophils are more effective than macrophages in the eradication of cysts (Alizadeh *et al*., 2002).

1.10.2 Diagnosis methods for Acanthamoeba Keratitis

Different methods are currently used for the diagnosis of AK. Microbiological culture is one of the best-known methods used to identify *Acanthamoeba*. This approach involves placing a culture of corneal scrapings from patients suspected of having AK on a non-nutrient agar plate seeded with a lawn of live *Escherichia coli* to allow for the observation of motile trophozoites. However, the major drawbacks of this method are the low culture-positive rate of 68% (Yeh *et al.*,

2006) and the long incubation period which often causes a delay in the diagnosis of AK (Bharathi *et al*., 2006). The use of quantitative polymerase chain reaction (qPCR) as a diagnostic tool has increased over the last two decades and the majority of more recent studies have used this technique for the diagnosis of AK due it is sensitivity, which ranges from 77% to 88% (Goh *et al*., 2018, Mathers *et al*., 2000, Pasricha *et al*., 2003). Moreover, PCR testing is faster and more sensitive than cultures, often returning a result within days instead of weeks, but similar to cultures, false negative results and misdiagnoses can occur. *In vivo* confocal microscopy (IVCM) is a clinical diagnostic method which is often used for diagnosing AK, as it has shown higher sensitivity values, ranging between 56 and100 % (Chidambaram *et al*., 2016, Hau *et al*., 2010, Tu *et al*., 2008, Kanavi *et al*., 2007).

The main advantages of IVCM are that it is non-invasive and gives a rapid diagnosis, but the major shortcomings are the potential difficulties in distinguishing pathogenic organisms from host cells, and diagnostic accuracy depends on the experience of the investigator (Hau *et al*., 2010). Formerly, three distinct methods were utilized to diagnose AK in patients: microscopy, a culture of corneal scrapings and qPCR. Outcomes indicated that qPCR is the most analytically-sound method, with a sensitivity of 89.3% compared to microscopy and corneal scrapings which had a sensitivity value of 21.8% (Khairnar *et al*., 2011). A delay in the diagnosis of AK of less than 18 days between the onset of symptoms and the start of anti-amoebic treatment results in improved visual acuity and eliminates the need for urgent penetrating keratoplasty (Claerhout *et al*., 2004).

1.10.3 Risk factors of Acanthamoeba Keratitis

Most cases of AK are related to poor hygiene and improper handling of contact lenses, in addition to other risk factors (Herz *et al*., 2008, Khan, 2006). In contact lens wearers, more than 85% of AK cases are associated with individual behaviours. AK has been observed more often in young males compared to other users of contact lenses, which might be due to poor personal hygiene, inadequate handling and care of their lenses or lens storage cases (Niederkorn *et al*., 1999). Several factors associated with AK infection, including swimming or showering while wearing contact lenses and using a chlorine solution as a disinfectant for

cleaning contact lenses and failing to wash hands before handling (Carnt *et al*., 2018). Furthermore, the type of lens could generate risk factors for AK, It has been reported that the use of silicone hydrogel contact lenses while swimming in a home pool and with an inability to clean contact lenses with an appropriate disinfection solution has resulted in an infection of *Acanthamoeba* (Stapleton *et al*., 2009).

1.11 *Acanthamoeba* act as reservoir for other pathogens

Acanthamoeba has been shown to harbour *Legionella pneumophila,* which is linked with Legionnaires' disease (Gast *et al*., 2011). Legionnaires' disease is significant due its capacity to cause bacteria to persist in *Acanthamoeba* spp. within hot water tanks and within buildings such as hospitals (Muraca *et al*., 1990). Several other pathogens have also been shown to survive intracellularly within *Acanthamoeba,* including *Escherichia coli* O157, which causes diarrhoea (Barker *et al*., 1999), *Vibrio cholera,* which causes Cholera (Thom *et al*., 1992), *Helicobacter pylori, a* causative agent for gastric ulcers (Winiecka-Krusnell *et al*., 2002) and *Mycobacterium avium*, which causes respiratory infections (Steinert *et al*., 1998). These bacterial pathogens do not simply survive intracellularly but they multiply within them and this can lead the bacteria to spread throughout the environment, evade chemotherapeutic drugs and produce infections (Khan, 2006). *Acanthamoeba* can act as a reservoir for many other pathogens and may aid their persistence in the environment. The protection of these other pathogens from biocides increases as a result of their intercellular location within *Acanthamoeba.* It has been demonstrated that PHMB produces a 4–log reduction in *L. pneumophila* in 6 hours but there has been shown to be only a 1–log reduction for those contained within *Acanthamoeba* (Barker *et al*., 1992). It has also been demonstrated that, at between 40 and 50°C, *Acanthamoeba* provides 10–fold resistance while 100-fold resistance is obtained at between 60 – 80°C (Storey *et al*., 2004).

1.12 Adhesion of *Acanthamoeba* and bacteria on contact lenses

The worldwide standard ISO 19045 sets out the microbiological requirements and testing methods for products as well as procedures for the hygienic administration of contact lenses (Standardization, 2015). The International Organization for

Standardization (ISO) evaluated certain disinfection agents against *Acanthamoeba* spp. and also assessed *Acanthamoeba* encystment caused by contact lens care products (Standardization, 2015). Contact lens cases can be considered as a reservoir for different types of gram-positive and negative bacteria which can lead to microbial keratitis in contact lens wearers. It has been estimated that the level of bacterial contamination in contact lens cases is higher than 50% (Wu *et al*., 2015). A case study was conducted at Moorfields Eye Hospital, UK, to assess the risk factors for microbial keratitis (MK) in different types of contact lenses and it has been shown that daily disposable contact lenses are associated with a higher risk of MK compared to rigid lenses and silicone hydrogel lenses with a lower risk of MK (Dart *et al*., 2008). Pervious *in vitro* study conducted by Dutta and Willcox (2013) showed that the bacterial adhesion to contact lenses is associated with a range of conditions, including contact lens material, the size of initial inoculum, nutritional content of media and the duration of incubation which played a crucial role in the bacterial adhesion to two types of contact lenses the hydrogel etafilcon A and silicone hydrogel senofilcon A.

Besides, the chemical and physical characteristics of contact lens material have an impact on bacterial adhesion to the lenses as the water content of hydroxyethylmethacrylate (HEMA)-based lenses and their iconicity entail in the binding of bacteria to contact lenses. The greater numbers of bacteria have been correlated with the hydrophobicity of silicone hydrogel lenses in comparison with the HEMA-based lenses (Dutta *et al*., 2012). *Acanthamoeba* showed greater binding in contact lens wearers compared with those who do not wear contact lenses. Previously, it was shown that fluorescein isothiocyanate-labelled lectins (including mannose, glucose, galactose, *N*-acetyl-glucosamine, *N*-acetylgalactosamine and *N*-acetyl neuraminic acid) bind to a range of sugars in soft contact lenses, see Figure 1.10. It is believed that the difference between wearers and non-wearers in terms of *Acanthamoeba* trophozoite binding is related to the presence of these saccharides on the surface of contact lenses (Klotz *et al*., 1987). A potential explanation for the increased binding of *Acanthamoeba* in contact lens wearers is that the molecules could serve as receptors for *Acanthamoeba* trophozoites leading to an increase in the ability of the organism to bind to the contact lenses.

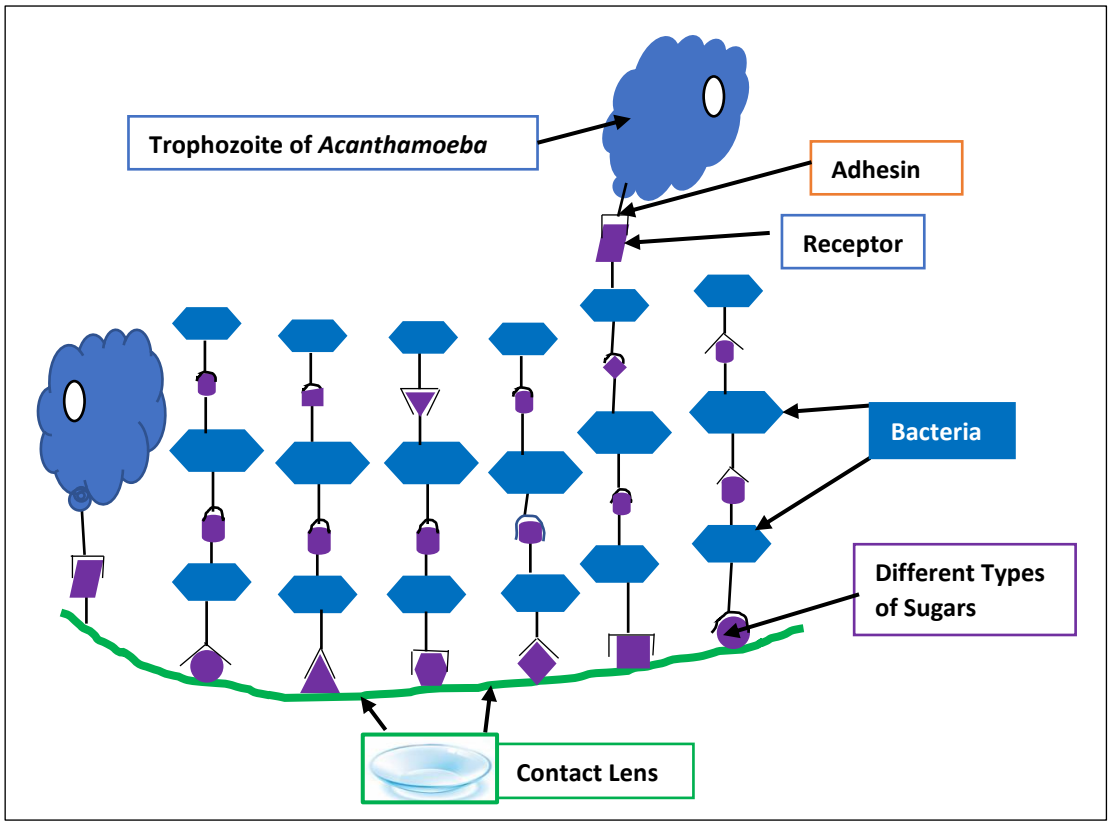

Figure1-10: The adhesion of *Acanthamoeba* trophozoites and bacteria to the surface of the contact lens. Their ability to adhere is increased by presence of various sugars on the lens. Regenerated from Khan (2009a).

1.13 Cellulose biosynthesis

Adrenaline can promote the encystment of *Acanthamoeba* by activation an adrenergic receptor and one of the key biochemical processes is the biosynthesis of cellulose leading to the formation of the cyst wall. The cellulose biosynthesis pathway is found in plants, bacteria and some protists, including *Acanthamoeba* and *Balamuthia*. Cellulose is believed to be responsible for biocidal resistance in *Acanthamoeba* (Garajová *et al*., 2019). The cellulose synthesis inhibitors (CSI) used in this study are herbicides that target cellulose synthesis in plants. The 2,6-dichlorobenzonitrile (DCB) is very effective against broadleaf and grass weeds in plants of different types, such as *Tussilago*, *Aegopodium*, *Rumex* and *Equisetum spp*. (Beynon and Wright, 1972),), whereas isoxaben has been used to control broadleaf weeds in several species of plants, for instance, speedwell (*Veronica* spp.), purple deadnettle (*Lamium purpureum* L.), violets (*Viola* spp.) and burning

nettle (*Urtica urens* L.) (Chandran and Derr, 1998). Cellulose biosynthesis in plants occurs in the plasma membrane forming rosettes, which represent a transmembrane system Figure 1.11. Cellulose synthase (CESA) protein complexes appear to be produced in the Golgi apparatus and then transferred by exocytosis to the plasma membrane (Somerville, 2006). The CESA complexes in the primary cell wall in the plant *Arabidopsis thaliana* are combined with three types of CESA subunits called CESA1, CESA3 and CESA6 (Persson *et al*., 2007). Cellulose cell walls in plants and *Acanthamoeba* are considered to be structural support. Both plants and *Acanthamoeba* are susceptible to herbicide glyphosate [*N*-(phosphonomethyl) glycine] and this herbicide can kill most of the plant species by targeting the Shikimate pathway (Tzin and Galili, 2010). Likewise, glyphosate was reported to be capable of inhibiting *Acanthamoeba castellanii* growth by the same pathway (Henriquez *et al*., 2015).

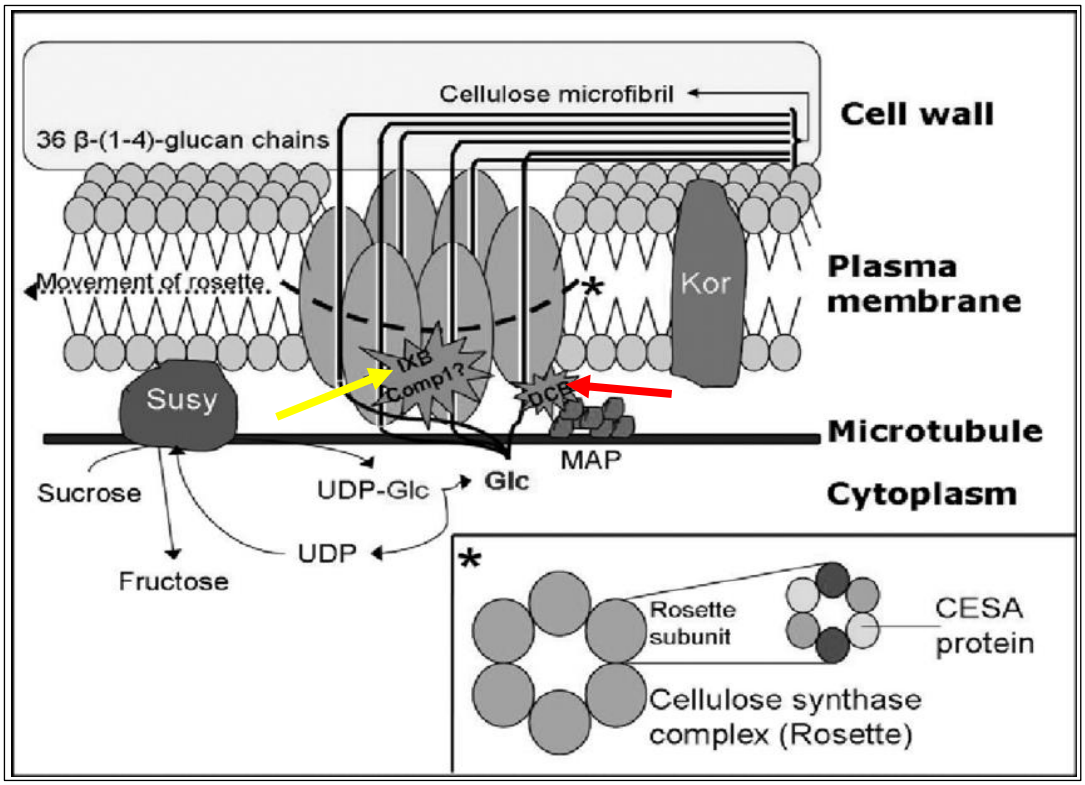

Figure1-11: Diagram showing the cellulose synthase complex for rosette in plants. Each subunit acts as segment comprising six CESA proteins which result in 36 distinct proteins in the rosette. Isoxaben targets CesA protein (yellow arrow), 2,6-dichlorobenzonitrile targets cellulose synthase (red arrow). Adapted from (Acebes *et al*., 2010).

Other proteins could also be involved in the formation of the cellulose synthase complex in plants, such as sucrose synthase, which breaks down to yield glucose and fructose (Amor *et al*., 1995). Another protein used in cellulose biosynthesis is membrane-anchored endo-β-(1-4)-glucanase, known as KORRIGAN (Nicol *et al.*, 1998). This protein plays an crucial role in reducing cellulose crystallinity (Takahashi *et al.*, 2009). The process of microfibril formation in the cellulose of plants is spilt into three stages: firstly, UDP glucose is utilised as the donor substrate; the next stage is the polymerisation of glucose into β-(1,4)-glucan chains and the final stage is the crystallisation of β-(1,4)-glucan chains into a cellulose microfibril (Peng *et al*., 2002). There are three different enzymes involved in cellulose biosynthesis in *Acanthamoeba*, including glycogen phosphorylase, UDP-glucose pyrophosphorylase and cellulose synthase, and the expression of these enzymes increases during encystment in *Acanthamoeba* (Moon and Kong, 2012). In *Acanthamoeba* cyst walls, the biosynthesis of cellulose begins by degrading the glycogen in the cytoplasm through catalytic action of glycogen phosphorylase, releasing glucose-1-phosphate. Consequently, UDP (uridine diphosphate) glucose is produced from glucose-1-phosphate and UTP (uridine triphosphate) catalysed by UDP-glucose pyrophosphorylase. The glucose residues are polymerized into individual glucan chains which are translocated across the cytoplasmic membrane by the action of cellulose synthase. Glucan chains are likely to be assembled into elementary fibrils connected into microfibrils (Garajová *et al*., 2019).

1.14 Drug targets for treatment of Acanthamoeba Keratitis

The biguanide drugs including PHMB and chlorhexidine, are the most effective drugs used to treat the AK infection, as they target the cell membrane of *Acanthamoeba* and lead to disruption and eventually cell death . There is a growing body of scientific literature which identifies a variety of anti-*Acanthamoeba* drugs, and these drugs have been categorised according to drug target and mode of action as presented in Table 1.3 below. The main challenge in AK therapy is the resistance of cysts to most antimicrobials. The cyst wall consists partly of cellulose and this has been the target of *in vitro* studies. Efforts have been made to block the cellulose synthesis in *Acanthamoeba* using

cellulose synthesis inhibitors such as isoxaben and 2,6-dichlorobenzonitrile (DCB); concentrations of DCB as low as micromolar levels have prevented the transformation of *Acanthamoeba* trophozoites into cysts (Dudley *et al.*, 2007).

Table 1-3: Classify the anti-Acanthamoeba drugs according to the targets and mechanism of action

Category	Class	Drug	Mechanism of action	Reference
Causing damage in the membranes	Biguanide	Polyhexamethylenebiguanide (PHMB), alexidine and chlorhexidine digluconate	Both PHMB and chlorhexidine are positively charged and binding to the mucopolysaccharide plug of the ostiole and causing penetration of the amoeba. Subsequent, the drug binds to the phospholipid bilayer of the cell membrane which is negatively charged causing changes in structural and permeability, ionic leakage, cytoplasmic disruptions producing cellular damage and cell death.	(Khunkitti *et al*., 1997) (Lim *et al*., 2008a)
	Antibiotic	Antibacterial included polymyxin B and linezolid	Polymyxin B targets negatively charged membranes and disrupts the membrane integrity. Linezolid prevents the formation of subunits of ribosome.	(Schindler and Teuber, 1975) (Shinabarger *et al*., 1997)

Table 1.3 (continued)

Category	Class	Drug	Mechanism of action	Reference
	Fluoroquinolone/ Antibiotic	Moxifloxacin, Levofloxacin	Moxifloxacin and levofloxacin are an inhibitor of DNA gyrase, a type II topoisomerase and topoisomerase IV which they required for DNA replication.	(Aldred *et al*., 2014)
	Anaesthetic	Proxymetacaine, tetracaine, oxybuprocaine and Lidocaine	These drugs are inhibiting bacterial growth by disruption the cell membrane	(Kaewjiaranai *et al*., 2018)
Inhibitors of nucleic acids synthesis	Diamidine	Pentamidine isethionate, propamidine isethionate and hexamidine	Diamidines inhibit the synthesis of DNA, RNA, phospholipids and proteins in *Acanthamoeba*	(Siddiqui *et al.*, 2016)
	Amidoamine	Myristamidopropyl Dimethylamine (MAPD) and Palmitamidopropyl Dimethylamine (PAPD)	MAPD and PAPD prevent the synthesis of DNA, RNA in *Acanthamoeba*. MAPD cause extensive damage for the plasma membrane of bacteria	(Codling *et al*., 2003)
Protein synthesis inhibition	Aminoglycoside antibiotic	Tobramycin	This antibiotic binds to the 30s and 50s ribosome for most of the Gram-negative bacteria and blocking the formation of 70s complex. Consequently, mRNA no longer can be translated into protein and eventually lead to cell death	(Le goffic *et al*., 1979)

Table 1.3 (continued)

Category	Class	Drug	Mechanism of action	Reference
Ergosterol synthesis inhibition	Antifungal	Azoles agents included voriconazole, posaconazole and benznidazole. Amphotericin B Antifungal macrolide natamycin	Azoles targeting the ergosterol which is one of the major sterols in the membrane of *Acanthamoeba* Amphotericin B binds to the ergosterol in the membrane and this binding disrupts osmatic integrity of the cell membrane, causing leakage in the intercellular potassium Mg^{+2}, sugars and then cell death. Natamycin binds to ergosterol in the plasma membrane of fungal and this binding leads to inhibiting ergosterol synthesis.	(Cowen *et al*., 2015)
Intracellular targeting agents	Quaternary ammonium	Benzalkonium chloride ,benzethonium chloride, Benzethonium chloride, Hexadecylpyridinium chloride, Hexadecyltrimethylammonium bromide and Polyquaternium-1	These compounds producing enzymes and lead to denaturation of the key cell proteins and distraction of the cell membrane	(Gerba, 2015)
Enzymes acting agents	Antineoplastic	Miltefosine	Interfere with carrier proteins systems, leading to a reduction in essential nutrients, and eventually to cell death	(Croft *et al*., 2003)

1.15 Treatment of Acanthamoeba Keratitis

As detailed in section 1.1 of this chapter that the first line of medical treatment for AK is polyhexamethylene biguanide or chlorhexidine at a concentration of 0.02% (v/v), used as a single drug or in combination with diamidines (Papa *et al*., 2020). Other compounds that are used for AK treatment include antifungals, such as oral itraconazole, and the topical use of 0.1 % (v/v) miconazole (Ishibashi *et al*., 1990). Although the T4 genotypes of *Acanthamoeba* are now recognised as being associated with pathogenic strains, other genotypes have also been identified in severe cases of AK that are resistant to present medical therapy (Arnalich-Montiel *et al*., 2014). A recent *in vitro* study investigated the effect of preserved propamidine (Brolene®) eye drops compared with propamidine as a pure drug and the findings showed that the presence of the benzalkonium chloride (BAC) as preservative in (Brolene®) is solely responsible for an improvement in antimicrobial activity against trophozoites and cysts of *Acanthamoeba* (Heaselgrave *et al*., 2019). BAC has also been used in different concentrations as a preservative of various classes of drugs, including antibacterials, antifungals, antivirals, anti-inflammatories/anti-infectives (NSAIDs), corticosteroids and anti-allergics (Tu, 2014).

In most countries, the standard treatment strategy for AK consists of a combination of biguanides, either chlorhexidine or PHMB, with a diamidine (propamidine or hexamidine), given hourly, then decreased to 2-hourly, by day for 3–4 weeks and then adjusted for each patient individually (Dart *et al*., 2009). However, most of these drugs are associated with toxic side effects for the cornea and therefore, following this treatment, most patients need to undertake keratoplasty for therapeutic purposes when the infection reaches an advanced stage (Dart *et al*., 2009). Antifungal agents, such as voriconazole and posaconazole, have been shown to have great activity against *Acanthamoeba* trophozoites and cysts *in vitro* (Iovieno et al., 2014a). Also, voriconazole has been given as an oral treatment for patients who are infected with persistent stromal AK and this antifungal has eradicated the stromal AK infection (Tu *et al*., 2010). Furthermore, cellulose synthesis inhibitors were found to be promising as a treatment for *Acanthamoeba*. They make the organism hidden within susceptible to existing chemotherapeutic agents by damaging the cyst wall. This therapy

should also be used in combination with other compounds to improve its potency, resulting in better treatment of *Acanthamoeba in vitro* (Lakhundi *et al*., 2015).

1.16 The aims and objectives of this project

Part 1: The aim is to develop novel therapeutic agents for the treatment of Acanthamoeba Keratitis

The specific objectives for this aim are described below:

I. Screening of new drugs along with existing ones against both trophozoites and cysts of *Acanthamoeba castellanii* (ATCC 50370) and *Acanthamoeba polyphaga* (ATCC 30461) to determine the minimum trophozoite inhibtory Concentration (MTIC), minimum trophozoite amoebicidal concentration (MTAC) and minimum cysticidal concentration (MCC) values.
II. Test a variety of topical ophthalmic drugs against both species of *Acanthamoeba*, the drugs including anaesthetics used in the corneal scrapes, empirical antibiotics/antivirals which are often given prior to an AK diagnosis.
III. Conduct toxicity studies against human epithelial cell line to define the toxicity of the drugs.
IV. Perform time kill analysis to determine the kinetics of cysticidal activity
V. Assess the antimicrobial activity for different drugs in combination against trophozoites.

Part two: The aim is to investigate the receptor involved in the encystment and examine the effect of cellulose synthesis inhibitors (CSI) on the formation of cyst and protocyst stages

The certain objectives for this aim are specified below:

I. Test several agonists and antagonists on the transformation of trophozoite of *Acanthamoeba* spp. into cyst to demonstrate which drug can bind to the receptor in *Acanthamoeba.*

II. Assess the effect of agonists and antagonists on the conversion of trophozoite into protocyst which is the new stage of *Acanthamoeba.*

I. Test different concentrations of (CSI) including isoxaben and 2,6-dichlorobenzonitrile in their own and in combination on the conversion of trophozoites into cyst and protocyst forms.

Part three: The aim is to study and confirm the potential existence of a 3rd life cycle stage pseudocyst/protocyst of *Acanthamoeba*

The particular objectives for this aim are stated below:

I. Prepare solutions of standard sugars for LC/MS analysis for the purposes of comparison.

II. Carry out acid hydrolysis and LC/MS for cellulose/chitin as standards to compare with cyst and protocyst samples.

III. Perform enzymatic treatments for cysts and protocysts to digest the intercellular elements.

IV. Do acid hydrolysis and LC/MS analysis for cyst and protocyst walls to determine the sugar types.

Chapter Two

Materials and Methods

Chapter 2: Materials and Methods

This chapter is describing the methodological approaches taken in this study.

2.1 Chemicals

All chemicals and most of the materials used in this study were obtained from VWR, (Lutterworth, Ltd, U.K.).

2.2 Media and solutions

Two methods have been used for sterilizing the solutions or media including autoclaving at 121°C for 15 min or by the passage of solution through a 0.2 µm filter.

2.3 Test organisms

Table 2-1: The organisms and cells which were used for the drug sensitivity, time kill, toxicological testing, encystment studies, cellulose biosynthesis investigations and sugars analysis.

Amoebae	Strain
Acanthamoeba castellanii	ATCC 50370
Acanthamoeba polyphaga	ATCC 30461
Acanthamoeba castellanii	ATCC 30868
Human cell line	Strain
Human epithelial cell type 2	ECACC #86030501
Gram – negative bacteria	Strain
Escherichia coli	ATCC 8739

Acanthamoeba castellanii (ATCC 50370), *A. polyphaga* (ATCC 30461) and *A. castellanii* (ATCC 30868) were obtained from the American Type Culture Collection (ATCC) (LGC Standards, Teddington, U.K.). The human cell line was obtained from the European Collection of Cell Cultures (Centre for Applied Microbiology and Research, Salisbury, U.K.).

2.4 Cultivation of *Acanthamoeba* spp.

The cryopreserved cells of the trophozoites were recovered from the freezing at -80°C and thawing them rapidly in a 37 °C water bath. Once melted, the suspension was added to a fresh, prewarmed Ac#6 growth medium in 25 cm^2 small culture flasks and incubated at a temperature of 32°C. The medium was replaced with a fresh Ac#6 medium after 6 hours to reduce any toxicity caused by the DMSO. After 24 hours of incubation, the cells were viewed under an inverted microscopy to check the confluency level of the cells and if the cells fully confluence then they were sub-cultured in 75cm^2 medium culture flasks and incubated at 32°C for 24 hours. In order to grow a sufficient number of cells for the experiments, the cells of the trophozoites were sub-cultured in large flasks 182.5cm^2 and incubated at 32°C for a further 24 hours.

The ingredients for this medium were based on a study by Hughes and Kilvington (2001), and comprised 20 g Biosate peptone (SLS, Nottingham, U.K.), 5 g glucose, 0.3 g potassium dihydrogen phosphate (KH_2PO_4), 100 µL vitamin B_{12} and 3 mL L-methionine per 800 mL of distilled water. The pH of the medium was adjusted to pH 6.5 – 6.6 with 1 M aqueous sodium hydroxide (NaOH) and then made up to 1000 mL and the resulting solution was aliquoted into 250 mL portions and autoclaved. Finally, a concentration of penicillin at 100 U/mL and 0.1 mg/mL was added to the medium and the completed medium was stored at room temperature and used within 1 week.

2.5 The cryopreservation of *Acanthamoeba* spp.

The trophozoites of the *Acanthamoeba* species were grown in an Ac#6 growth medium and the cells were pelleted by centrifugation at 500 × *g* for 5 min and the final pellets were resuspended in an Ac#6 growth medium containing 5% (v/v) dimethyl sulfoxide (DMSO) as a cryoprotectant and placed in a 1 mL cryotube. The cells were then placed in a Mr Frosty freezing container containing propan-2-ol and placed in a -80°C freezer for 2 days. The propan-2-ol plays a major role in slowing the rate of freezing to 1°C /min and assisting to decrease cellular damage.

2.6 The cryopreservation of the human epithelial cell line

A Dulbecco's Minimum Essential Medium (DMEM) was used to grow the human epithelial cells type 2 (Hep2) and the cells were harvested by centrifugation at 500 × *g* for 5 min. The resulting pellets were resuspended in a DMEM medium containing 5% (v/v) DMSO as a cryoprotectant and the mixture was transferred to a 1 mL cryotube. The Hep2 cells were then placed in a Mr Frosty freezing container containing propan-2-ol and stored in a freezer at -80°C for 48 hours. The main reason for using the propan-2-ol was to reduce the speed of freezing to 1°C /min and to aid the decline of cellular damage. The Hep2 cells were recovered by taking the cell vails out of the -80°C freezer and melting them quickly in a 37°C water bath. Once the medium had dissolved, the suspension of human epithelial cells was added to a fresh, prewarmed DMEM medium and incubated at 37°C, then a fresh medium was substituted after 6 hours in order to decrease any toxicity resulting from the DMSO.

2.7 The preparation of *Acanthamoeba* mature cysts in Neff's medium

Cysts were produced using the Neff's encystment medium (NEM) method as described by Kilvington and Lam (2013). Trophozoites were seeded in 182.5 cm^2 tissue culture flask at a density of 1 × 10^5 cells/mL in 50 mL of Ac#6 growth medium and incubated for 48 hours at 32 °C. The trophozoites were harvested by centrifugation at 500 × *g* for 5 min and washed three times with a ¼ strength Ringer's solution (SLS, Nottingham, U.K.) and the final pellet was resuspended in 50 mL of NEM at a density of 5 × 10^5 cells/mL in a 75 cm^2 tissue culture flask. The cultures were incubated in an orbital shaking incubator at 32°C and 120 rpm for 7 days. The cysts were harvested for antimicrobial testing after 7 days of incubation in NEM and the maturity of the cysts was then confirmed using an inverted microscopy before harvesting. The flask sides were swabbed to remove any adhering cysts which washed three times with a ¼ strength Ringer's solution and centrifuged at 1000 x *g* for 10 min. The pellet was adjusted to 5 × 10^6 cells/mL using a modified Fuchs Rosenthal haemocytometer and the batch of cysts was stored between 4 – 8°C and tested within 14 days.

2.8 The preparation of *Acanthamoeba* mature cysts in NNA medium

A different approach was used for the preparation of a smaller number of cysts, the non-nutrient agar (NNA) method, which was adapted from Kilvington *et al.* (1990). The non-nutrient agar plates containing 2.5% (w/v) agar and 1 tablet of ¼ strength Ringer's per 500 mL of deionised water were autoclaved then poured on the plates and allowed to dry overnight. *Acanthamoeba* trophozoites were centrifuged at 500 x *g* for 5 min and the resulting pellet was resuspended in the *Escherichia coli* (ATCC 8739) stock. 2-3 drops of the *Acanthamoeba* trophozoite *E. coli* suspension were spotted onto the lawn of *E. coli*.

The plates were then incubated at 32°C for 7 – 10 days for the encystment process to complete. During the 7 – 10 day period, the plates were examined on a regular basis to check for any contamination and as well as to verify amoeba replication. Once the *Acanthamoeba* have spread out to cover the whole plate, the bacterial lawn is quickly depleted triggering the amoeba to encyst. For harvesting, roughly 20 mL of ¼ strength Ringer's solution was poured onto the agar surface and firm pressure was applied in a circular motion using the rubber teat. The resulting cyst suspensions were collected and pelleted by centrifugation at 1000 x *g* for 10 min and washed three times in a ¼ strength Ringer's solution to remove any trace of *E. coli*. Finally, the cells were resuspended in a ¼ strength Ringer's solution and the cell density was assessed by microscopy using a modified Fuchs Rosenthal haemocytometer and adjusted to 5×10^6 cells/mL and then stored between 2 – 8°C and tested within two weeks.

2.9 The preparation of *Acanthamoeba* protocysts in Neff's medium plus 0.5% propylene glycol

For the transformation of trophozoites into the form of protocysts, The method used for this experiment has been adapted from the work carried out by Kilvington *et al.* (2008). The trophozoites were harvested by shaking the flask and centrifuging it at 500 x *g* for 5 min. The pellet was then washed three times with a ¼ strength Ringer's solution. The resulting pellet of trophozoites was resuspended in 30 mL of Neff's + 0.5% (v/v) propylene glycol (Neff's-PG) in a 75 cm^2 tissue culture flask and the flask was placed in an orbital shaking incubator

at 32°C and 120 rpm for 2 hours. The trophozoite cells were rapidly differentiated into protocysts as confirmed using an inverted microscopy. The protocysts were harvested by swabbing the flask sides to remove any adherent protocysts and washed three times with a ¼ strength Ringer's solution and centrifuged at 1000 × *g* for 5 min. The final pellet was adjusted to the desired concentration in a ¼ strength Ringer's solution and then stored between 2 – 8 °C and tested within 7 days.

2.10 The preparation of *Escherichia coli* stock for the *Acanthamoeba* food source

E. coli (ATCC 8739) was cultured on a tryptic soy agar (TSA) plate and incubated overnight at 37 °C to allow for colony growth. Single colonies of *E. coli* were selected and placed in 100 mL of tryptic soy broth (TSB) in a 182.5 cm^2 tissue culture flask and incubated overnight at 37°C and at 120 rpm in an orbital shaking incubator. The *E. coli* suspension was aseptically transferred to two 50 mL polypropylene tubes and centrifuged at 3000 × *g* for 30 min to pellet the bacteria. The supernatant was then discarded, and the pellet was washed by re-suspending in a ¼ strength Ringer's solution. The pellet of *E. coli* was washed twice in order to remove the growth broth and to avoid further multiplication of the bacteria. The final *E. coli* pellet was resuspended in 10 mL of ¼ strength Ringer's solution and then stored between 2 – 8°C and used within 14 days.

2.11 *In vitro* evaluation of drug antimicrobial activity

Many compounds were screened during the present study in order to identify their potential antimicrobial activity using a method similar to that used by Elder *et al.* (1994). To set up the microtitre plate to assay antimicrobial sensitivity against trophozoites and cysts of *Acanthamoeba*, 200 µL of a 1000 µg/mL solution of test compound was prepared in a suitable diluent and added to column 1, rows A – H, of the microtitre plate, as presented in Figures 2.1 and 2.2. The remainder of the wells were filled with 100 µL of a ¼ strength Ringer's solution. Serial 2-fold dilutions were then carried out across the plate from column 1 to column 11, giving a wide range of drug concentrations from 1000 to 0.98 µg/mL, which is double the preferred final concentration. Column 12 contained only a ¼ strength Ringer's solution to serve as a control. Cysts were resuspended in a ¼ strength Ringer's solution and trophozoites were resuspended in Ac#6 growth medium

and added to the wells to give a final concentration of 1×10^4 cells/mL and change the drug concentration from 500 to 0.49 μg/mL. The microtitre plates were transferred to the 32°C incubator and, after 24 hours of incubation, the plates containing trophozoites were examined, as described in section 2.12. The plates containing cysts were checked after 48 hours for further experimentation as detailed in section 2.13.

2.12 Evaluation of drug activity against trophozoites

After completing the trophozoites assay, the wells of the microtitre plate (Figure 2.1) were checked after 24 hours to see if there was any contamination. Furthermore, after 48 hours the plates were examined under an inverted microscopy at x 100 objective and x 200 objective. By making a comparison of the test compounds with the control wells, it was possible to determine the following:

- **Minimum Trophozoite Inhibitory Concentration (MTIC)**: The concentration at which there is approximately 50% inhibition of *Acanthamoeba* trophozoite replication compared with the controls.
- **Minimum Trophozoite Amoebicidal concentration (MTAC):** The concentration at which all the *Acanthamoeba* trophozoites were non-motile, round or lysed.

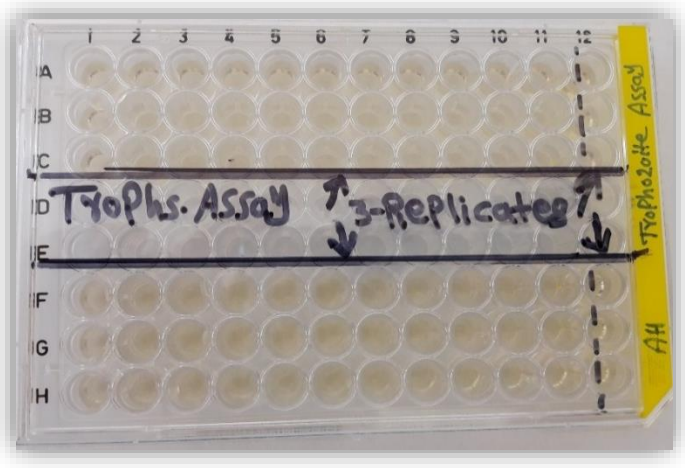

Figure 2-1: Microtitre plate showing the three replicates used for *in vitro* drug sensitivity testing against trophozoites of *Acanthamoeba* spp.

2.13 Evaluation of drug antimicrobial activity against cysts

In order to assess the antimicrobial activity for the drugs against *Acanthamoeba* cysts, an assay was performed by exposing the cysts to the drugs for 48 hours. After 2 days of incubation at 32°C, the wells of the microtitre plate (Figure 2.2) were aspirated using a Vacusip to remove the drug and leave only the cysts adhering to the bottom. The wells were refilled with a ¼ strength Ringer's solution and allowed to stand for 15 min for the cysts to reattach and the liquid was again aspirated as before. This process was then repeated three times to ensure the drug was fully removed from the wells. An *E. coli* suspension at an OD_{520} of 0.1 – 0.2 was then added to each well of the plates. The plates were incubated at 32°C and examined daily under an inverted microscopy for up to 7 days to determine whether still-viable cysts had excysted and the trophozoites replicated in the wells. It was possible to determine the following:

- **Minimum Cysticidal Concentration (MCC)**: The lowest concentration of test solution that results in no encystment or trophozoite replication.

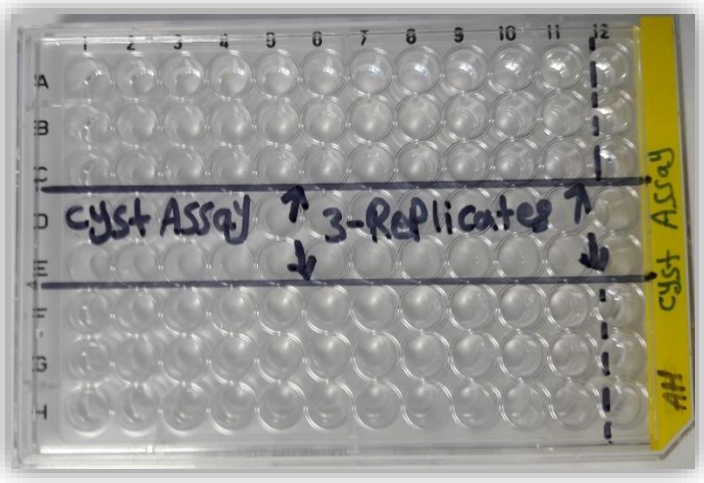

Figure 2-2: Microtitre plate showing the three replicates used for the in vitro drug sensitivity testing against cysts of *Acanthamoeba* spp.

2.14 Time kill testing for *Acanthamoeba* spp.

All of the compounds which showed great antimicrobial activity against cysts of *Acanthamoeba* spp. were further tested using a time-kill method, based on an approach described by Kilvington (2004). The microtitre plate was set up for this assay by adding 180 μL of 0.1 % (v/v) Tween 80 and 0.35 % (w/v) Soybean Lethicin neutralisers to columns A and H with the remaining wells being filled with 180 μL of a ¼ strength Ringer's solution as shown in Figure 2.3. The purpose for used neutralisers (Tween 80 and Soybean Lecithin) is to neutralise the drugs in order for growth, replication and encystment to occur.

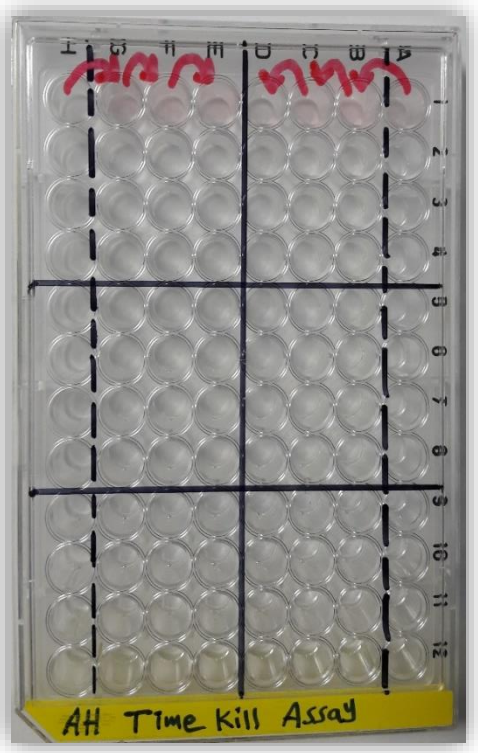

Figure 2-3: Microtitre plate showing the time-kill assay for cysts or trophozoites of *Acanthamoeba* spp.

All the time kill experiments were carried out in triplicate in 50 mL polypropylene centrifuge tubes, with each tube containing 5 mL of an appropriate test solution or control. The cysts or trophozoites of *Acanthamoeba* were added to each centrifuge tube at a density of (50μL of 5 × 10^6 cells/mL) at time 0 and vortexed. At time intervals of typically 0, 1, 2, 4, 6 and 24 hours, 20 μL aliquots containing around 1000 cells were removed from the centrifuge tube in groups of four wells

and transferred to the wells of a microtitre plate containing a 0.1% (v/v) Tween 80 and 0.35 % (w/v) Soybean Lecithin neutraliser solution. Once the aliquots were neutralised, a series of 10-fold dilutions were performed. For example, the contents of A1 – 4 were transferred into B1 – 4 then into C1 – 4 and then into D1 – 4. Each well was then seeded with a 1:40 dilution of the *E. coli* stock and the plates were incubated at 32°C for 7 – 14 days. On a daily basis, the plates were examined for encystment or trophozoite growth using an inverted microscopy.

The number of viable organisms at each time point was calculated using the Spearmann-Karber formula, as illustrated below:

Log_{10} organism = $(X_0 – (d/2) + d\ (\Sigma\ r_i / n_i)$

Where: X_0 = $\log_{10}$ of the lowest dilution at which all test inocula are positive

d = $\log_{10}$ of the dilution factor

n_i = number of test inocula used at each dilution

r_i = number of positive test inocula out of n_i

$(\Sigma\ r_i / n_i) = \Sigma$ (P) sum of the percentage of positive tests showing a 100 % positive result

Here are two examples to clarify the Spearmann-Karber formula above:

Example-1: If 4 wells contained viable cysts or trophozoites at the 10^{-1}, 10^{-2}, 10^{-3} dilutions and 1 out of 4 at the 10^{-4} dilution, using the formula below:

Log_{10} organism = $(X_0 – (½) + 1\ (4/4+4/4+4/4) = 5600$

Example-2: If 4 wells contained viable cysts or trophozoites at the 10^{-1} dilution and 1 out of 4 at the 10^{-2} and 10^{-3} dilutions, using the formula below:

Log_{10} organism = $(X_0 – (½) + 1\ (4/4+1/4+1/4) = 100$

2.15 Data analysis for time kill experiments

To assess the reduction in viable *Acanthamoeba,* the decrease in numbers was plotted as log viability at each of the time points and the reduction was determined relative to the log viability at time zero using the formula below:

Log reduction = log T_n – log T_0

Where T_n indicates the viable cell count at any given experimental time point

T_0 indicates the initial viable cell count

A template was created in excel spreadsheet to perform the delta log calculation and graphs were generated.

2.16 Cultivation of human epithelial cells

Human epithelial type 2 (Hep2) cells were grown in a medium consisting of Dulbecco's Minimum Essential Medium (DMEM) (SLS, Nottingham, U.K.) plus 10 % (v/v) foetal bovine serum, 2 mM L-glutamine. 100 U/mL and 0.1 mg/mL respectively of penicillin/streptomycin were added to the final concentration. The Hep2 cells were cultured in 75 cm^2 tissue culture flask and placed in an incubator at a temperature of 37°C and in an environment of 5 % CO_2. The DMEM medium was added to the flasks and the medium was replaced every 3 – 4 days. When the medium became acidic, it converted to a yellowish colour. Depending on the confluence of the Hep2 cells, they were either sub-cultured or maintained for up to 5 days in a maintenance medium which was the same as the growth medium, but it contained only 3% (v/v) foetal bovine serum.

2.17 Sub-culture and maintenance of human epithelial cells

Initially, the DMEM medium was gently removed from the flask and the monolayer was washed once in 10 mL of Dulbecco's phosphate buffered saline (DPBS) (SLS, Nottingham, U.K.), pre-warmed to 37°C, in order to wash away any residual buffer. 3 mL of trypsin-EDTA solution was then added to the flask. The cells were incubated for 4–5 min at room temperature in the trypsin-EDTA (Sigma, Dorset, U.K.) solution until rounding of the cells was observed when viewed under an inverted microscopy. The flask was then knocked against the hand to remove the cells from the flask. Confirmation that this had occurred was again achieved by viewing under an inverted microscopy. The flasks were re-incubated, and knocking was repeated if all cells had not detached. The resulting cell suspension was then divided into fresh medium at a ratio of between 1:2 and 1:4, depending on the level of confluence of the monolayer, and placed in the growth medium before being put back in the incubator at 37 °C.

2.18 *In vitro* toxicology assay

Experimental human epithelial type 2 cells were used to establish the toxic effects of the novel and existing antimicrobial compounds tested in this study. *In vitro,* the microtitre plates were seeded with human epithelial cells at a density of 2 × 10^4 cells/mL and the plates were incubated at 37°C and 5% CO_2 for 48 hours or until the monolayer reached 80 – 90% confluence. The spent growth medium, comprising 10% (v/v) foetal bovine serum, was removed from all the plates by aspirating the wells using a Vacusip and then replaced with 100 μL of maintenance medium. The antimicrobial compounds were serially diluted across the microtitre plates in a series of 2–fold dilutions, accomplishing a range of concentrations as done for the testing of antimicrobial activity in *Acanthamoeba* as described in sections 2.11 – 2.13.

To identify the toxic effects of the test compounds, two different methods were used in the present study. The first of these is called the CellTiter 96® AQ_{uous} One Solution Cell Proliferation Assay (Promega, Southampton, U.K.), which is a colorimetric method. This approach combines a tetrazolium MTS compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt) with PES (Phenazine ethosulfate), an electron-coupling agent. If the cells are metabolically active, the MTS compound is reduced by the NADPH or NADH enzymes, generating a coloured formazan product. Subsequently, 5 μL of the CellTiter 96® assay was added to each well of the microtitre plate and the plates were incubated overnight at 37°C and 5% CO_2. The quantity of coloured product produced in a colorimetric approach was measured spectrophotometrically in an MRX microplate reader at 490 nm and compared with a predetermined calibration curve. Furthermore, toxicity was also established using a light inverted microscopy in a similar way to the *Acanthamoeba* drug sensitivity assay. The following elements of the toxicological profile was estimated:

- **Minimum Inhibitory Concentration (MIC)**: The minimum concentration at which inhibition of cell division and localised detachment of the monolayer occurred, relative to the control well.

- **Minimum Cytotoxic Concentration (MCT):** The minimum concentration at which all human epithelial cells were rounded and lysed with general monolayer detachment.

Chapter Three

Evaluation the effect of topical ophthalmic agents and anti-*Acanthamoeba* drugs on *Acanthamoeba* spp. viability

Chapter 3: Evaluation the effect of topical ophthalmic agents and anti-*Acanthamoeba* drugs on *Acanthamoeba* spp. viability

3.1 Introduction

3.1.1 Current medical treatments for Acanthamoeba Keratitis

The medical therapy for Acanthamoeba Keratitis (AK) involves the topical administration of a biguanide, either 0.02% (v/v) polyhexamethylene biguanide (PHMB) or 0.02% (v/v) chlorhexidine as monotherapy (Papa *et al*., 2020) or in combination with 0.1% (w/v) of propamidine or hexamidine is still unlicensed (Dart *et al*., 2009). It has been reported that chlorhexidine and PHMB are the most effective agents against both the trophozoites and cysts of the *Acanthamoeba* species (Lim et al., 2008b, Lorenzo-Morales *et al*., 2013). A recent publication has shown that octenidine hydrochloride is more active against cysts and trophozoites then PHMB and chlorhexidine (Heaselgrave *et al.,* 2019).

Octenidine hydrochloride is derived from pyridine that has a broad spectrum of activity and it can be used as an effective antimicrobial against most gram-positive and gram-negative bacteria (Hübner *et al*., 2010). Early research conducted by Sedlock and Bailey (1985) showed that octenidine (OCT) is more active than chlorhexidine (CHLX) and at lower concentrations of 2.0 µM and within only 15 min of exposure, it caused more than a kill of 99.99% for a range of species of bacteria, including *Staphylococcus epidermidis, Proteus mirabilis, Streptococcus pyogenes, Klebsiella pneumoniae, Escherichia coli, Pseudomonas aeruginosa, Serratia marcescens,* and fungi of *Candida albicans*. However, a higher concentration of chlorhexidine at 40 µM was required to achieve a similar antimicrobial activity and exposure time with OCT at 99.99%. At present there are around 4.1 million contact lens wearers in the United Kingdom (Efron, 2016). In spite of the sight threatening risk with AK, in most series, it accounts for <5% of all contact lens associated with other forms of keratitis (which includes bacterial, fungal and herpes simplex keratitis). The reported incidence rates in contact lens users are 1 to 2 per million in the United States to 17 to 20 per million in the United Kingdom (Radford *et al*., 2002).

Galarreta *et al*. (2007) investigated the fungal keratitis related to the contact lenses over 12 years and found that the rate of this keratitis is 4.5 cases per year and this is a relatively low incidence compared to the another study conducted by Ong *et al*. (2016) in Moorfields Eye Hospital which reported a higher rate of fungal keratitis associated with contact lenses between 2007 and 2014 period was 14 cases per year. A much higher global incidence of HSV keratitis is approximately 1.5 million, involving 40,000 new cases of severe monocular visual impairment or blindness every year (Farooq and Shukla, 2012). AK is very rare and the latest research from a tertiary hospital in the United Kingdom reported an incidence rate of only 2.3% for *Acanthamoeba* over a 12-year period from over 1500 keratitis cases (Tan *et al*., 2017).

Many antimicrobial agents have been tested against *Acanthamoeba* species and most of these agents do not warrant any further investigations. Povidone-iodine has been found to be an effective agent against the cysts of *Acanthamoeba* (Sunada *et al*., 2014). Furthermore, alexidine has been shown to be active at a concentration of 10 µg/mL against trophozoites of *Acanthamoeba castellanii*, *Acanthamoeba polyphaga*, and *Acanthamoeba rhysodes.* However, a concentration 10 times higher (100 µg/mL) of this agent was required against cysts of *A. castellanii*, *A. polyphaga*, and *A. rhysodes* to achieve a complete kill (Hassan Alizadeh and Cavanagh, 2009). Despite the significant advances in medical treatment, rapid diagnosis and combination therapy, the mean treatment period for patients suffering from AK may be more than 5 months, with surgical operations possibly needed in 15% of cases and loss of vision of up to 6/8 or less may occur in more than 30% of patients (Pérez-Santonja *et al*., 2003, Larkin *et al*., 1992, Chidambaram *et al*., 2016).

3.1.2 Anaesthetic, antibiotic and antiviral drugs

Three topical anaesthetics, proxymetacaine 0.5%, tetracaine 1% and oxybuprocaine 0.4%, were tested *in vitro* against four different strains of bacteria: *Staphylococcus epidermidis, Staphylococcus aureus, Pseudomonas aeruginosa*, and *Streptococcus pneumoniae*. The proxymetacaine did not cause any inhibition of bacterial growth and exhibited a MIC of >5000 µg/mL. In contrast, the other two anaesthetics showed inhibition on all bacterial strains used in the review study (Pelosini *et al*., 2009). These agents were tested in this study to assess

their effect on the viability of *Acanthamoeba*, as they used to anesthetise the patient eye for a corneal scrape. Another investigation compared the effect of commercial eye-drops containing oxybuprocaine 0.2 %, tetracaine 0.4 % and lidocaine 1 % on the inhibition of bacterial growth and the results indicated that lidocaine at a concentration of 1 % showed no impact on the growth of bacteria compared to the other anaesthetics tested (Labetoulle *et al*., 2002). It is very important for an ophthalmologist to use a suitable anaesthetic before a corneal scrape is performed. Empirical treatment with antibiotic fluoroquinolone drugs or other biocides prior to diagnosis showed that they could have an impact on the viability of *Acanthamoeba*. Bacterial keratitis is more likely and therefore empirical treatments with antibiotics is started initially. In addition to antimicrobial drugs, many ophthalmic preparations utilise benzalkonium chloride (BAC) as a preservative and it has been shown that BAC is highly toxic to *Acanthamoeba* (Tu *et al*., 2013). In a case study performed by Wolf *et al.* (2009), the topical anaesthetic proparacaine and the antibiotic moxifloxacin were used to treat a patient who suffered from photophobia and redness caused by the contact lens. After the treatment had been performed, the outcomes of the study showed that the cultures for bacteria and fungi were negative. However, the results confirmed that the culture of *Acanthamoeba* was positive in this case (Wolf *et al*., 2009). The possible reason for not detecting any bacteria is related to using the antibiotic moxifloxacin

It was found that fluorescein and topical anaesthetics could interfere with the real-time polymerase chain reaction (real-time PCR) which is called quantitative PCR (qPCR) to detect the herpes virus and *Acanthamoeba* resulting in false negative results (Goldschmidt *et al*., 2006). One potential reason for the low sensitivity of culture-based diagnostics for *Acanthamoeba* might be associated with the previous use of a topical therapy, such as anaesthetics and antibiotics, which was applied to the cornea prior to the corneal scrape being performed on the patient. The topical antiviral aciclovir has been used at a concentration of 3% as an ointment for the treatment of the herpes simplex virus (HSV) and it showed more activity *in vivo* then valaciclovir (Choong *et al*., 2010). Trifluorothymidine is another antiviral agent which acts as an inhibitor of thymidylate synthetase and a solution of only 1% of this antiviral was found to be active against HSV

(Wilhelmus, 2007). HSV is more common that AK and in many cases the patient is misdiagnosed with HSV and treated with anaesthetics and antibiotics. We attempted to see if these drugs can negatively impact on AK diagnosis by reducing viability.

3.1.3 Aim and objectives of this chapter

The aim of this chapter is to evaluate the antimicrobial activity of various agents on the viability of *Acanthamoeba* and to assess the toxicity on the human epithelial cell line. The objectives of this chapter are as follows:

I. To test different groups of agents, including topical anaesthetics, antivirals, antibiotics, diamidines and biguanides, against cysts and trophozoites of *Acanthamoeba* spp.
II. To evaluate the toxicity for these agents for human epithelial cell line.
III. To investigate the effect of a number of agents on the morphology changes of Neff's cysts of *A. castellanii* by inverted light microscopy and transmission electron microscopy imaging.

3.2 Materials and Methods

3.2.1 Preparation of organisms

The *Acanthamoeba castellanii* (ATCC 50370) and *Acanthamoeba polyphaga* (ATCC 30461) were grown and prepared according to the methods detailed in chapter 2, sections 2.4 – 2.8.

3.2.2 Drug sensitivity testing assays

The drugs were diluted in the appropriate solvent and assayed to determine their activity against trophozoites and cysts using the microtitre plate assay as outlined in chapter 2, section 2.11 – 2.13. The Minimum Trophozoite Inhibitory Concentration (MTIC), the Minimum Trophozoite Amoebicidal Concentration (MTAC) and the Minimum Cysticidal Concentration (MCC) were determined for each of the compounds.

Table 3.1: List of drugs evaluated for their antimicrobial activity against *Acanthamoeba castellanii* (ATCC 50370) and *Acanthamoeba polyphaga* (ATCC 30461) and for their toxicity against human epithelial cell line.

Drug	Class/Use	Solubility	Supplier
Proxymetacaine	Anaesthetic	Minims	Bausch & Lomb (London, U.K.)
Tetracaine	Anaesthetic	Minims	Bausch & Lomb (London, U.K.)
Oxybuprocaine	Anaesthetic	Minims	Bausch & Lomb (London, U.K.)
Lidocaine (+Fluorescein Na)	Anaesthetic	Minims	Bausch & Lomb (London, U.K.)
Fluorescein sodium	Use as diagnostic tool of corneal infections	Minims	Bausch & Lomb (London, U.K.)
Levofloxacin (Oftaquix®)	Fluoroquinolone/ Antibiotic	Minims	Bausch & Lomb (London, U.K.)
Levofloxacin (Pure drug)	Fluoroquinolone/ Antibiotic	H_2O	Bausch & Lomb (London, U.K.)
Moxifloxacin (Moxeza®,)	Fluoroquinolone/ Antibiotic	Minims	Bausch & Lomb (London, U.K.)
Chloramphenicol (eye drops)	Antibiotic	Minims	Bausch & Lomb (London, U.K.)
Chloramphenicol (presertive free)	Antibiotic	H_2O	Bausch & Lomb (London, U.K.)

Table 3.1 (continued)

Drug	Class/Use	Solubility	Supplier
Acyclovir	Antiviral	H_2O	Sigma (Dorset, U.K.)
Trifluorothymidine	Antiviral	H_2O	Bausch & Lomb (London, U.K.).
Propamidine (Pure drug)	Diamidine	H_2O	Sigma (Dorset, U.K.)
Propamidine (Brolene®)	Diamidine	H_2O	Sanofi (Reading, U.K.)
Hexamidine (Pure drug)	Diamidine	H_2O	Sigma (Dorset, U.K.)
Hexamidine (Desomedine®)	Diamidine	H_2O	Bausch & Lomb, Montpellier, France
Pentamidine (Pure drug)	Diamidine	H_2O	Sigma (Dorset, U.K.)
Polyhexamethylene Biguanide	Polymeric biguanide	H_2O	Arch Biocides (Castleford, U.K.)
Chlorhexidine	Biguanide	H_2O	VWR (Lutterworth, U.K.)
Octenidine hydrochloride	Quaternized pyridine	H_2O	Schulke & Mayr, Norderstedt, Germany
Alexidine	Biguanide	H_2O	Sigma (Dorset, U.K.)
Povidone iodine	Antiseptic	H_2O	VWR (Lutterworth, U.K.)
Benzalkonium Chloride	Cationic surfactant	H_2O	Sigma (Dorset, U.K.)
Phenylmercuric Nitrate	Antimicrobial preservative	H_2O	Sigma (Dorset, U.K.)

3.2.3 Toxicological testing

After determining the antimicrobial activity for all compounds against trophozoites and cysts of *Acanthamoeba* spp. A different *in vitro* method was used in order to identify the toxicity of each of the compounds for the human epithelial cell line. The human epithelial cell line was cultured, maintained and prepared as outlined in chapter 2, sections 2.16– 2.18. This approach is particularly useful in determining the minimum inhibitory concentration (MIC) and minimum cytotoxic concentration (MCT) for each compound and this method could be used on the same basis as for *Acanthamoeba* drug sensitivity testing.

3.2.4 Inverted light microscopy study of *Acanthamoeba* cysts

Primary investigations were undertaken using inverted light microscopy to examine the effects of topical ophthalmic preparations or other biocide solutions on the Neff's cysts of *Acanthamoeba castellanii* (ATCC 50370). The Neff's cysts of *A. castellanii* were treated with tetracaine 1% and preserved chloramphenicol 0.5 % or solutions composed of similar concentrations to those utilised in ophthalmic preparations (PHMB 0.02 %, unpreserved chloramphenicol 0.5 %, benzalkonium chloride 0.05 mg/mL and povidone-iodine 5 %). The cysts were incubated with ¼ strength Ringer's solution as a control and then the samples were placed in incubator at 32 °C for 1 hour. Subsequently, the samples were removed from the incubator and washed three times with ¼ strength Ringer's solution and centrifuged at 1000 x *g* for 5 min in order to eradicate the agents. The final pellets of cysts were fixed overnight at 4°C with 2.5 % (v/v) glutaraldehyde buffered with 0.1 M HEPES at pH 7.2. Examination of the samples was conducted by using a pipette to place 10 µL of the fixed cysts on slides and overlaying with a cover slip. The cover slip was then sealed to the slide using a DPX mounting medium (Sigma, Dorset, U.K.) to prevent the suspension from drying out. The slides were then viewed using inverted microscopy at x200 magnification.

3.2.5 Preparation of the cysts of *Acanthamoeba* for study using transmission electron microscopy (TEM)

Neff's cysts of *Acanthamoeba castellanii* (ATCC 50370) were investigated using transmission electron microscopy (TEM). The Neff's cysts were exposed to the test formulations using the following topical ophthalmic preparations: preserved chloramphenicol 0.5 % and tetracaine 1% or a solution made up to the same concentrations as those used in ophthalmic preparations (povidone-iodine 5%, unpreserved chloramphenicol 0.5%, benzalkonium chloride 0.05mg/mL and PHMB 0.02%). The Neff's cysts were exposed to the test formulations at 32 °C for 1 hour and the control cysts were exposed to ¼ strength Ringer's solution. The agents were removed by washing the cysts with ¼ strength Ringer's solution and centrifuged at 1000 x *g* for 5 min. The resulting pellets were fixed overnight at 4°C with 2.5 % (v/v) glutaraldehyde buffered with 0.1M HEPES at pH 7.2.

3.2.6 TEM process for the *Acanthamoeba* cysts

The fixed Neff's cysts were centrifuged at 0.8 rpm for 5 min and then the speed was increased to 2.6 rpm for 10 min. The samples were washed twice in 0.05 M HEPES and centrifuged at 800 rpm for 10 min. The TEM process was performed as previously described by Glauert (1975). The cysts were postfixed in 1% osmium tetroxide and 1.5% potassium ferricyanide in 0.05 M HEPES for 90 min and then washed in 0.05 M HEPES for 20 min and a further wash was performed in the same buffer for 30 min and then stored at 4°C. The pellets were dehydrated in 70% and 90% ethanol for 30 min and then washed three times in analytical grade ethanol for 20 min. The samples were infiltrated with propylene oxide plus Spurr's resin for a different periods of time and embedded in Spurr's resin twice for 3 hours. The samples were then polymerised at 60°C for 16 hours. Finally, the samples were divided into sections approximately 70 nm thick using a Leica UC7 ultramicrotome, collected on copper mesh grids and then stained with 2% aqueous uranyl acetate for 30 min, followed by 5 min in Reynolds lead citrate. The samples were delivered to the TEM microscopy facility in College of Science and Engineering, University of Leicester, UK. The samples were viewed on a JEOL JEM-1400 TEM with an accelerating voltage of 100kV.

3.3 Results

The findings in this chapter are comparing the antimicrobial activity for a range of different agents including anaesthetics, antibiotics, antivirals, diamidines and biguanides on the viability of trophozoites and cyst of *Acanthamoeba* spp. and also the toxicity against the human epithelial cell line.

3.3.1 The topical anaesthetics

Lidocaine inhibited the trophozoites of both species of *Acanthamoeba* at the higher concentration range of 312 – 625 µg/mL, whereas the other anaesthetics, including proxymetacaine, tetracaine and oxybuprocaine, demonstrated MTIC at a lower concentration range of 9.75–39 µg/mL. The MTAC for the proxymetacaine, tetracaine and oxybuprocaine was in the range 19.5 – 250 µg/mL. However, against *A. polyphaga* and *A. castellanii,* the MTAC for lidocaine was recorded at 312 and 1250 µg/mL, respectively.

Cysticidal activity of proxymetacaine, tetracaine and oxybuprocaine was found in the range 39 – 250 µg/mL, whereas the MCC values of lidocaine were observed at the higher concentrations of 1250 and 10000 µg/mL against *A. polyphaga* and *A. castellanii* respectively. Furthermore, proxymetacaine, tetracaine and oxybuprocaine showed toxicity for the human epithelial cell line in the range 39 – 156 µg/mL, while no toxicity was observed for lidocaine up to 5000 µg/mL against the Hep2 cells. The lidocaine MINIMS formulation contained fluorescein sodium and so, as a control, this was tested individually. The 2% concentration was found to be non-toxic (as shown in Table 3.2).

Table 3.2: Efficacy of topical anaesthetics and fluorescein sodium against *Acanthamoeba* spp. trophozoites and cysts, and toxicity to a human epithelial cell line (Hep2).

In vitro drug sensitivities (µg/mL)								
	A. castellanii (ATCC 50370)			*A. polyphaga* (ATCC 30461)			Hep2 cells	
Drug	MTIC*	MTAC**	MCC***	MTIC*	MTAC**	MCC***	MIC+	MCT++
Proxymetacaine	39	156	156	39	78	156	78	39
Tetracaine	9.75	19.5	39	19.5	39	78	312	156
Oxybuprocaine	31.3	250	125	15.6	125	250	250	125
Lidocaine (+Fluorescein)	312	1250	10000	625	312	1250	10000	5000
Fluorescein sodium (2%)	>10000	>10000	>10000	>10000	>10000	>10000	2500	1250

MTIC* minimum trophozoite inhibitory concentration, MTAC** minimum trophozoite amoebicidal concentration, MCC*** minimum cysticidal concentration, MIC+ minimum inhibitory concentration, MCT++ minimum cytotoxic concentration. What is interesting about the data in Table 3.2 is that tetracaine had a significant effect on the viability of the cysts and trophozoites of both tested species compared with other anaesthetics that assessed in this study.

3.3.2 The topical antibiotics and antivirals

Two fluoroquinolones, levofloxacin, both as a pure drug and preserved with benzalkonium chloride (BAC), and moxifloxacin (Moxeza®), were tested in the current study and the findings are shown in Table 3.3. The levofloxacin (Oftaquix®) formulation demonstrated MTIC between 78 – 156 µg/mL and the MTAC was between 156 – 312 µg/mL against both species of *Acanthamoeba*. The activity was reduced when levofloxacin (as a pure drug without BAC) was tested, with the values ranging from 312 up to 1250 µg/mL against both species of *Acanthamoeba.* This amounts to a difference of a factor of 4 in magnitude when compared with commercial ophthalmic preparations. When levofloxacin (Oftaquix®) was tested against cysts, cysticidal activity was observed at 625 µg/mL. This compared to cysticidal activity within the range of 2500 – 5000 µg/mL for levofloxacin in the pure drug version and moxifloxacin (Moxeza®).

Levofloxacin (Oftaquix®) and moxifloxacin were found to be toxic for the human epithelial cell line at concentrations of 39 and 156 µg/mL, respectively. Two versions of chloramphenicol were tested in this study: preservative-free chloramphenicol showed inhibitory activity against trophozoites at 39 to 312 µg/mL whereas chloramphenicol as a pure drug showed inhibitory activity between 312 and 625 µg/mL. The cysticidal activity for the preservative-free chloramphenicol was found to be at the same values for the trophozoites inhibitory which produced by the chloramphenicol as pure drug, however, the chloramphenicol as pure drug showed activity against cysts at a higher concentration of 1250 up to 2500 µg/mL.

Phenylmercuric nitrate, which is the preservative used in chloramphenicol, was tested in this study and its activity against trophozoites was observed in a concentration range of 1 – 3.9 µg/mL but activity declined against cysts at concentrations of 15.6 to 31.3 µg/mL. Benzalkonium chloride (BAC), which is typically added to ophthalmic preparations as a preservative, was also tested and the findings showed that it exhibited activity against trophozoites in the 1 – 7.8 µg/mL range and against cysts in the 7.8 – 15.6. µg/mL range (Table 3.3).

Two antiviral drugs, trifluorothymidine (TFT) and acyclovir, were assessed for their antimicrobial activity in the current study. TFT showed inhibitory activity against trophozoites at concentrations of between 312 – 625 µg/mL and amoebicidal activity at concentrations of 625 – 1250 µg/mL. The cysticidal activity was found at the highest concentrations of 2500 – 5000 µg/mL and the MCT for the human epithelial cell line was at 156 µg/mL. Aciclovir ophthalmic ointment (Zovirax®) could not be applied during *in vitro* testing due to its soft paraffin base and, therefore, a solution was made up from the pure drug and tested in the present study. Aciclovir showed inhibitory activity against trophozoites at concentrations of between 63 – 125 µg/mL and the MTAC occurred at 125 – 250 µg/mL against both species of *Acanthamoeba*. No activity was observed from this antiviral when tested against cysts at the highest concentration of >500 µg/mL and the toxicity for the human epithelial cell line occurred within the range 31.3 – 62.5 µg/mL (see Table 3.3).

Table 3.3: Efficacy of topical antibiotics, antivirals and preservatives against *Acanthamoeba* spp. trophozoites and cysts, and toxicity to a human epithelial cell line (Hep2).

In vitro drug sensitivities (µg/mL)								
	A. castellanii (ATCC 50370)			*A. polyphaga* (ATCC 30461)			Hep2 cells	
Drug	MTIC*	MTAC**	MCC***	MTIC*	MTAC**	MCC***	MIC+	MCT++
†Levofloxacin (Oftaquix®)	78	156	625	156	312	625	78	39
Levofloxacin (Pure drug)	312	1250	2500	625	1250	5000	156	78
Moxifloxacin (Moxeza®)	625	2500	2500	1250	2500	2500	312	156
Chloramphenicol (Pure drug)	312	625	2500	312	625	1250	1250	625
††Chloramphenicol (Generic)	78	312	625	39	156	312	1250	312
Aciclovir (Pure Drug)	63	125	>500	125	250	>500	62.5	31.3
†Trifluorothymidine	312	625	5000	625	1250	2500	312	156
Benzalkonium Chloride	3.9	7.8	15.6	1	1.95	7.8	62.3	31.3
Phenylmercuric Nitrate	1.95	3.9	31.3	1	1.95	15.6	7.8	3.9

MTIC* minimum trophozoite inhibitory concentration, MTAC** minimum trophozoite amoebicidal concentration, MCC*** minimum cysticidal concentration, MIC+ minimum inhibitory concentration, MCT++ minimum cytotoxic concentration. † Compound is preserved with benzalkonium chloride (0.005% w/v). †† Compound is preserved with phenylmercuric nitrate (0.002% w/v). From the data in Table 3.3, it can be seen that benzalkonium chloride as preservative enhanced the antimicrobial activity for levofloxacin and Trifluorothymidine. Likewise, the phenylmercuric nitrate as preservative increased the activity for chloramphenicol .

3.3.3 The diamidines

The antimicrobial activity of a range of diamidine compounds against the trophozoites and cysts of *Acanthamoeba* spp. and also their toxicity for the human epithelial cell line are presented in Table 3.4. Propamidine was tested as both a pure drug and in the Brolene® formulation (which contains BAC). Propamidine as a pure drug inhibited trophozoites at a concentration of 63 – 250 µg/mL compared to a range of 7.8 – 15.6 µg/mL for the Brolene® formulation. Amoebicidal activity was observed at a higher concentration range of 250 – 500 µg/mL for both species of *Acanthamoeba* in comparison with a range of 15.6 – 31.3 µg/mL for the Brolene® formulation. Both the pure drug and the Brolene® formulation showed limited to no activity against cysts of both species.

For pure drug propamidine, partial toxicity was observed at 250 µg/mL compared to 31.3 µg/mL for the Brolene® formulation. In this study, the same results were obtained when hexamidine as a pure drug and the desomedine® formulation were tested against trophozoites and cysts of both species and the human epithelial cell line. Pentamidine exhibited almost comparable activity to that of propamidine. BAC was relatively more effective than any of the diamidines tested in this study and showed activity against trophozoites at lower concentrations ranged from 1 – 7.8 µg/mL and cysts 7.8 – 15.6 µg/mL as range and greater cytotoxicity for the human epithelial cell line at MCT 31.3 µg/mL (Table 3.4).

Table 3.4: Efficacy of diamidine compounds against *Acanthamoeba* spp. trophozoites and cysts, and toxicity to a human epithelial cell line (Hep2).

In vitro drug sensitivities (µg/mL)								
	A. castellanii (ATCC 50370)			*A. polyphaga* (ATCC 30461)			Hep2 cells	
Drug	MTIC*	MTAC**	MCC***	MTIC*	MTAC**	MCC***	MIC+	MCT++
Propamidine (Pure drug)	62.3	250	>500	250	500	>500	500	250
†Propamidine (Brolene®)	7.8	15.6	500	15.6	31.3	250	62.3	31.3
Hexamidine (Pure drug)	7.8	62.3	250	7.8	31.3	250	125	62.3
Hexamidine (Desomedine®)	7.8	62.3	250	7.8	31.3	250	125	62.3
Pentamidine (Pure drug)	62.3	250	>500	125	250	>500	250	125

MTIC* minimum trophozoite inhibitory concentration, MTAC** minimum trophozoite amoebicidal concentration, MCC*** minimum cysticidal concentration, MIC+ minimum inhibitory concentration, MCT++ minimum cytotoxic concentration.

† Compound is preserved with benzalkonium chloride (0.005% w/v). It is apparent from the data in Table 3.4 that benzalkonium chloride as a preservative in the formulation of propamidine (Brolene®) is responsible for enhanced activity of this compound against trophozoites compared to the use of propamidine as a pure drug.

3.3.4 The biguanides

The effects of the biguanide compounds and povidone iodine on *Acanthamoeba* trophozoites and cysts, and their toxicity for the human epithelial cell line, are shown in Table 3.5. The concentrations at which inhibitory activity occurred for all of the biguanides tested in this study were between 0.5 – 1.95 µg/mL and the minimum amoebicidal concentration in trophozoites of both species was found to be in the range 1 – 15.6 µg/mL. Both octenidine and alexidine demonstrated greater cysticidal activity in the range 3.9 – 7.8 µg/mL, while cysticidal activity was reduced for PHMB and chlorhexidine in the range 7.8 to 31.3 µg/for both species. The toxicity of the biguanides against the human epithelial cell line occurred in the range 1 – 31.3 µg/mL, with PHMB having the highest value (minimum toxicity). Povidone iodine showed MTIC at 7.8 µg/mL and MTAC at 31.3 µg/mL for both species of *Acanthamoeba*, whereas the MCC for this compound ranged from 7.8 – 15.6 µg/mL and it was not toxic for the human epithelial cell line at a MCT of 125 µg/mL.

Table 3.5: Efficacy of biguanides and povidone iodine compounds against *Acanthamoeba* spp. for trophozoites and cysts and toxicity to a human epithelial cell line (Hep2).

In vitro drug sensitivities (µg/mL)								
	A. castellanii (ATCC 50370)			*A. polyphaga* (ATCC 30461)			Hep2 cells	
Drug	MTIC*	MTAC**	MCC***	MTIC*	MTAC**	MCC***	MIC+	MCT++
Polyhexamethylene Biguanide	1	3.9	15.6	1	7.8	7.8	62.3	31.3
Chlorhexidine	1	3.9	31.3	1.95	15.6	31.3	7.8	3.9
Octenidine hydrochloride	1	1.95	7.8	0.5	1	3.9	3.9	1.95
Alexidine	1	1.95	3.9	1.95	7.8	7.8	1.95	1
Povidone iodine	7.8	31.3	15.6	7.8	31.3	7.8	250	125

MTIC* minimum trophozoite inhibitory concentration, MTAC** minimum trophozoite amoebicidal concentration, MCC*** minimum cysticidal concentration, MIC+ minimum inhibitory concentration, MCT++ minimum cytotoxic concentration. The most interesting aspect of the findings in Table 3.5 is that octenidine hydrochloride demonstrated a higher antimicrobial activity against cysts and trophozoites in comparison to other tested biguanides and povidone iodine.

3.3.5 Inverted light microscopy observation

The effects of different agents on the morphology of Neff's cysts were demonstrated using inverted light microscopy and the findings of these investigations are shown in Figure 3.1. The healthy Neff's cyst in ¼ strength Ringer's solution as control is shown in Figure 3.1.A. In healthy cysts, a thick cyst wall can be seen surrounding the encysted trophozoite. When the cyst was incubated with tetracaine, the cyst walls were disrupted (Figure 3.1.B). No changes were observed in morphology when the cysts were exposed to preserved chloramphenicol, propamidine pure drug, and unpreserved chloramphenicol (Figures 3.1.C, D, and F). When the cyst was incubated with BAC (0.05 mg/mL), povidone iodine (5% w/v) and PHMB (0.02 %), there was clear damaged and destruction in the endocyst and the walls of the ectocyst (Figures 3.1.E, G and H).

3.3.6 Transmission electron microscopy study

In order to confirm the preliminary results of the inverted light microscopy and to provide a detailed explanation of how these agents affect the intracellular elements of the cyst, Neff's cysts of *Acanthamoeba* were exposed to various agents and changes in the morphology of the cyst were observed using TEM imaging. The results are presented in Figure 3.2. A Neff's cyst exposed to ¼ strength Ringer's solution as control and a healthy cyst can be seen in Figure 3.2.A.

The healthy cyst has a thick cyst wall surrounding the encysted trophozoite. In the plasma membrane, the trophozoite links with the endocyst wall taking up the whole space available inside the cyst. No change occurred in the cytoplasm and the nucleus is clear as a rounded structure including mitochondria and lysosomes. When the cyst was treated with tetracaine, the nucleus disappeared and the cytoplasm was completely full of micelles, which were produced by the breakup of the nuclear membrane (Figure 3.2.B). No alterations were observed in the intracellular organisation of either the cytoplasm or the nucleus when the cysts were exposed to preserved chloramphenicol, propamidine pure drug and unpreserved chloramphenicol (Figures 3.2.C, D, and H). Obvious damage occurred to the plasma membrane of the encysted trophozoite after exposing the cyst to BAC (0.05 mg/mL) and it shrank away from the walls of the endocyst.

Moreover, the nucleus was not visible, and the number of cytoplasmic micelles increased, a clear indication of membrane damage (Figure 3.2.E). Following the exposure of the cyst to povidone iodine (5% w/v) and PHMB (0.02%), intensive damage occurred to the plasma membrane of the encysted trophozoite which shrank away considerably from the walls of the endocyst. No defined nuclear structures were observed and there were significant numbers of micellar aggregations inside the cyst, indicating considerable plasma membrane damage in the encysted trophozoite (Figures 3.2.F and 3.2.G).

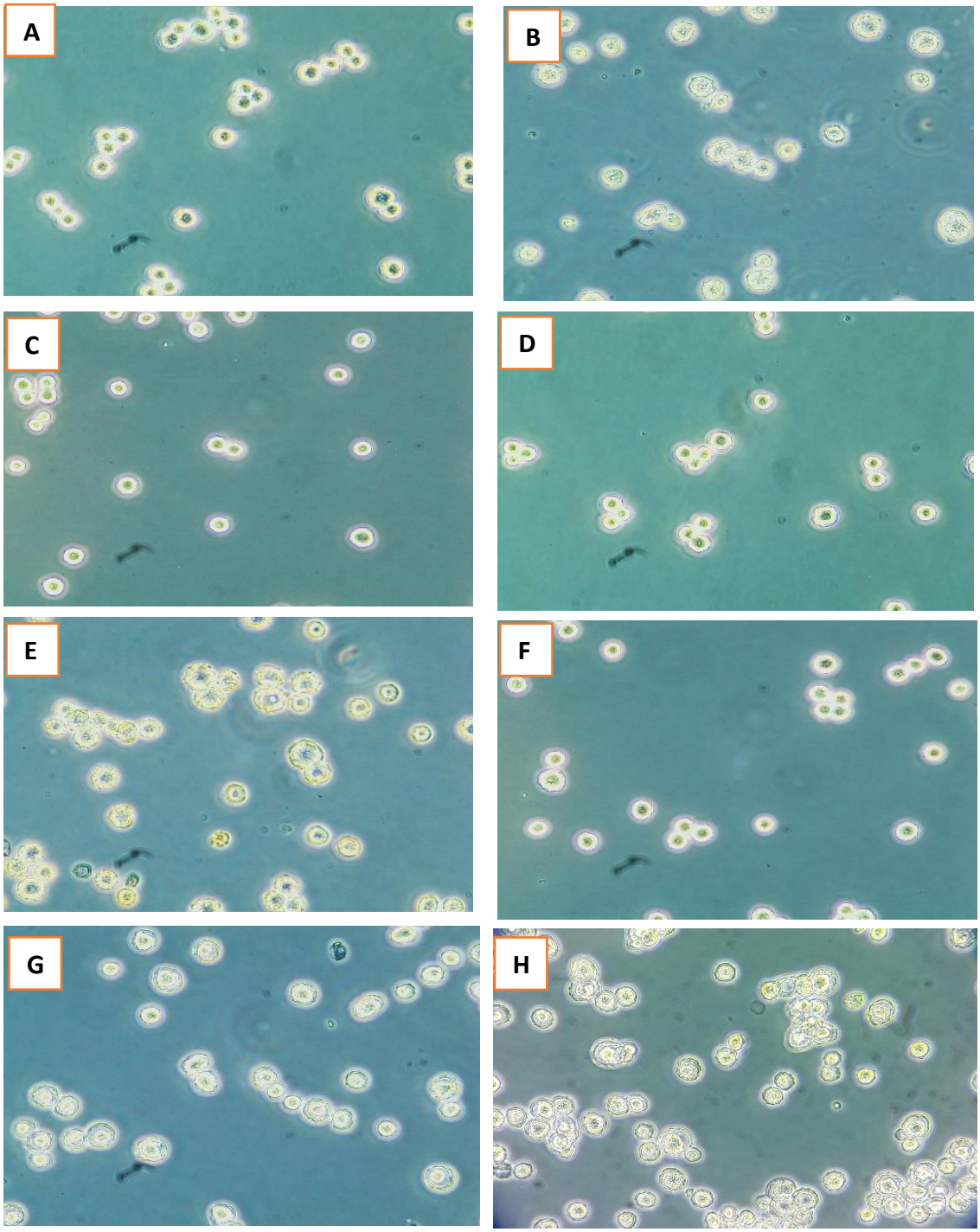

Figure 3-1: Inverted light microscopy images of *Acanthamoeba* Neff's cysts following 1 hour of exposure to the subsequent 7 agents. (A) Untreated Neff's cysts; (B) treated with 1 % tetracaine; (C)treated with 0.5 % chloramphenicol (preserved); treated with 0.1 % propamidine; (E) treated with 0.05 mg/mL benzalkonium chloride; (F) treated with 0.5 % chloramphenicol (unpreserved); (G) treated with 5 % povidone- Iodine; (H) treated with 0.02 % PHMB. Magnification is x200.

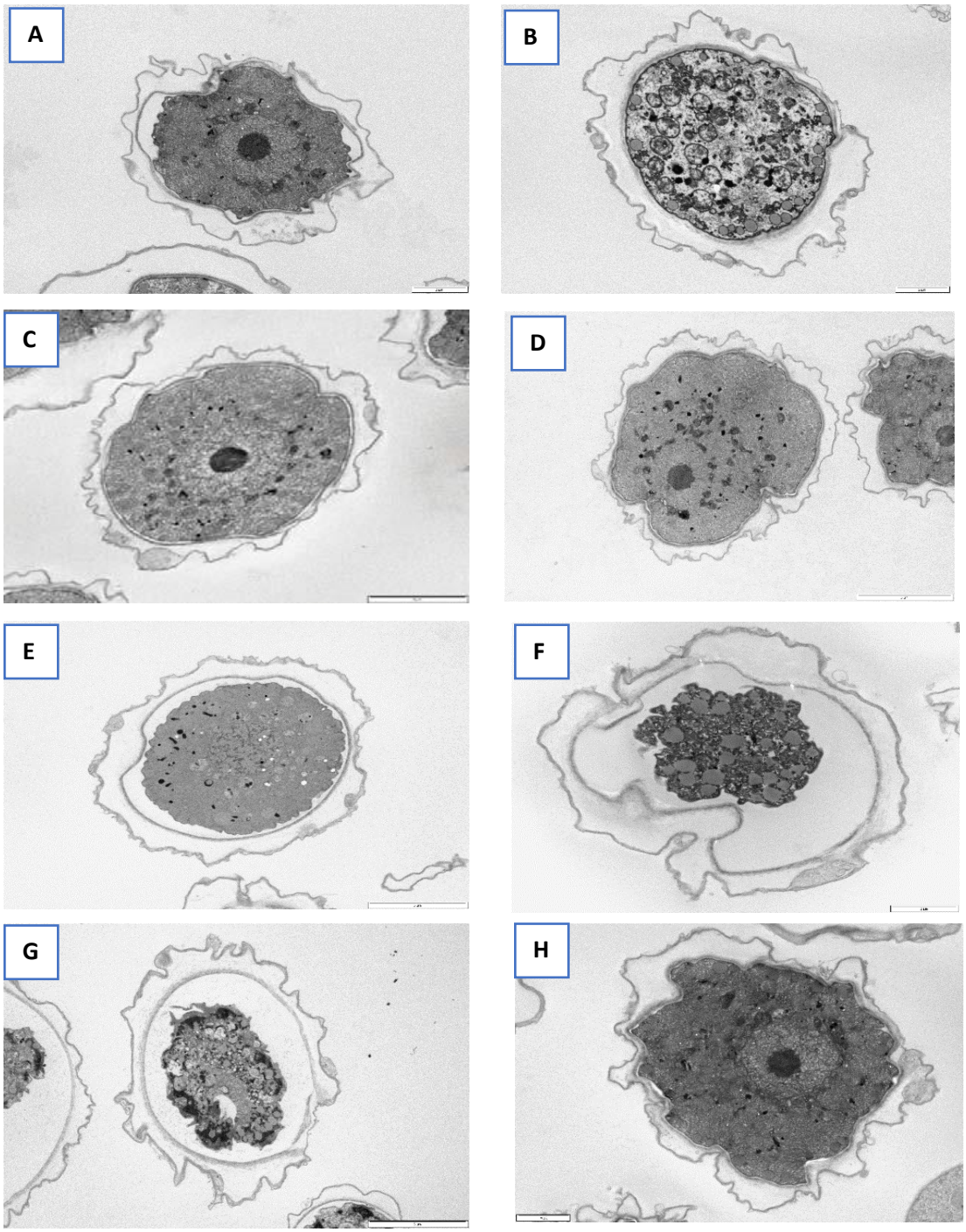

Figure 3-2: Transmission electron microscopy images of Acanthamoeba cyst after 1 hour exposure to the following 7 agents: (A) untreated healthy cyst as control; (B) treated with 1 % tetracaine; (C) treated with 0.5 % preserved chloramphenicol; (D) treated with 0.1 % propamidine pure drug; (E) treated with 0.05 mg/ml benzalkonium chloride; (F) treated with 5 % povidone iodine; (G) treated with 0.02 % PHMB; (H) treated with 0.5 % unpreserved chloramphenicol. Bar= 2 µm.

3.4 Discussion

In this chapter, a variety of compounds have been tested *in vitro* which often given to AK patients including anaesthetics used to anaesthetise the eye, antibiotics applied in empirical treatment, antivirals used if misdiagnosed with herpes simplex keratitis and biocides including biguanides and diamidines.

3.4.1 The effects of topical anaesthetics on the viability of *Acanthamoeba*

AK is a sight-threatening corneal infection and prompt diagnosis is the most important factor in achieving a better prognosis and outcome for vision. In order to differentiate AK infection from the other types of keratitis, determine the risk factors and clinical examination are essential. The clinical indicators of AK are ring infiltrate or disease being limited to the epithelium when compared with other keratitis, including bacterial and fungal (Mascarenhas *et al*., 2014). Nonetheless, in epithelial disease, and particularly when linked with dendritic-type lesions, incorrect diagnosis of *Acanthamoeba in vivo* can occur, resulting in other causes of keratitis, such as herpes simplex (HSV) keratitis, being reported (Radford *et al*., 2002, Stehr-Green *et al*., 1987). There is an association between the amount of viable *Acanthamoeba* found in a corneal scrape or biopsy and the inhibitory effect of using of topical agents, including anaesthetics and vital stains, such as fluorescein, before microbiological sampling is performed (Goldschmidt *et al*., 2006).

There have been relatively few studies relating to the inhibitory effect of topical anaesthetics with, one study in the literature showed that proparacaine (proxymetacaine) did not negatively affect the polymerase chain reaction (PCR) (Thompson *et al*., 2008). However, another study conducted by Goldschmidt *et al*. (2006) found that oxybuprocaine inhibited the real-time polymerase chain reaction (real-time PCR) in detecting *Acanthamoeba*. The current study found that lidocaine had a limited antimicrobial effect on the viability of *Acanthamoeba* and, at therapeutic concentrations, it did not exhibit significant antimicrobial activity against cysts and trophozoites for either species of *Acanthamoeba*.

Although the lidocaine tested in this study was combined with fluorescein, the findings from testing fluorescein as a single therapy showed that it had limited antimicrobial activity against cysts and trophozoites of *Acanthamoeba*. These results differed from those relating to other topical anaesthetics, especially tetracaine, which demonstrated greater antimicrobial activity against trophozoites and cysts of *Acanthamoeba.* This agent was also toxic for the human cell line. A comparable effect was observed by proxymetacaine and oxybuprocaine against *A. castellanii,* but for *A. polyphaga*, proxymetacaine showed significant amoebicidal activity but it also showed higher toxicity for the human cell line. Based on the findings of this study, it is proposed that the use of topical anaesthetics, in particular tetracaine, may have greater anti-amoebic effects and they could contribute significantly to the low sensitivity of culture detected in a corneal scrape.

3.4.2 Evaluating the activity of antibiotics and antivirals

There is a link between the number of viable *Acanthamoeba* cells identified from a corneal scrape and the inhibitory effect of empirical treatment with antibiotics prior to microbiological sampling being performed (Thompson *et al*., 2008). Based on the antibiotics protocol used, empirical treatment with third or fourth generation fluoroquinolones is frequently prescribed as a first therapy for the treatment of microbial keratitis because of their broad-spectrum antimicrobial activity (Marangon *et al*., 2004). Preserved levofloxacin (Oftaquix®) with BAC showed strong antimicrobial activity against both species of *Acanthamoeba*. However, when this antibiotic was tested as a pure drug, its antimicrobial activity against cysts and trophozoites declined. This finding is not surprising as the activity of this preserved antibiotic is related to the BAC constituent rather than the antibiotic itself. Furthermore, the testing of a preservative-free preparation of moxifloxacin (Moxeza®) did not show any significant effect against either species of *Acanthamoeba*.

A prior study by Thompson *et al*. (2008) did not find any negative effects on PCR amplification in *Acanthamoeba* with either gatifloxacin or moxifloxacin. The gatifloxacin tested in their study (Zymar; Allergan, Irvine, CA) was preserved with BAC but the moxifloxacin was self-preserved. Also, the authors did not test BAC as a monotherapy to examine its activity. The MTIC effect observed for both

antibiotics indicates that the effect of BAC on PCR in detecting *Acanthamoeba* DNA could be lower compared to the amoebicidal and cysticidal assays performed in the present study. There was a significant difference between the two chloramphenicol preparations: the preserved drug exhibited greater antimicrobial activity against both species of *Acanthamoeba,* hinting that the anti-amoebic effect may be associated with the preservative phenylmercuric nitrate. Moreover, the TEM images did not show much difference in morphology for the preserved and preservative-free versions of chloramphenicol.

Aciclovir and trifluorothymidine are antiviral drugs which showed good antimicrobial activity against trophozoites of both species but limited or no activity was observed in either agent against cysts. Aciclovir is widely used to treat HSV keratitis and, as AK is commonly misdiagnosed as HSV, the potential inhibitory impact on *Acanthamoeba* trophozoites would need to be borne in mind. BAC is commonly used as a preservative in various multi-dose ophthalmic preparations in order to avoid contamination of the formulation. Consequently, the empirical use of BAC preserved eye drops before the diagnosis of AK is expected to have a negative effect on the viability of the *Acanthamoeba* present on the cornea, which could be contributing to the ongoing reported low positive culture rate for AK from corneal scrapes.

3.4.3 The antimicrobial activity of diamidines

There was a clear difference in anti-amoebic activity for preserved propamidine (Brolene®), a common-over-the-counter (OTC), anti-infective ophthalmic preparation in the UK, compared with the pure drug propamidine. In the past, it has been demonstrated that the concentrations of BAC often used in ophthalmic medicines are very toxic to *Acanthamoeba* trophozoites. An *in vitro* study carried out by Tu *et al*. (2013) reported that BAC inhibited *Acanthamoeba* trophozoites at concentrations in the range 10 – 30 µg/mL and the findings of their study showed a 4.5 log reduction in the viability of trophozoites after 6.5 hours of exposure. Nevertheless, the current study is the first to show the effect of preparations containing BAC against the very resistant cystic stage of *Acanthamoeba*.

This study found that the MTIC, MTAC and MCC values of BAC were significantly lower than the concentration of BAC present in both Brolene® (0.05 mg/mL) and Oftaquix® (50 µg/mL) ophthalmic preparations. In fact, the current study has shown that the existence of the BAC preservative in propamidine (Brolene®) eye drops is likely to be exclusively responsible for the observed antimicrobial activity against trophozoites and cysts of both species. Early *in vitro* studies tested preserved propamidine (Brolene®) and the results showed that the MTAC values were 5 – 25 µg/mL and 0.49 – 0.97 µg/mL. However, in investigations by both Elder *et al*. (1994), Hay *et al*. (1994b), only the BAC-preserved Brolene® was tested without comparing it with propamidine as a pure drug.

To our knowledge, no attempt has been made to compare propamidine as a pure drug with propamidine (Brolene®) preserved with BAC. This study found a very significant difference in anti-amoebic activity, with MTAC values for Brolene® being in the range 15.6 – 31.3 µg/mL. These results are identical to those in the study by Hay et al. (1994b) and greater than the values found in the study conducted by Elder *et al*. (1994). This study was able to show that the anti-amoebic activity of Brolene® is associated with the existence of BAC in the formulation, as, when propamidine was tested without BAC, the MTAC values were significantly higher, in the 250 –500 µg/mL range. Moreover, the amount of BAC in Brolene® (50 µg/mL) is much higher than the MTAC observed in this study, which is 12.8 times greater than the concentration found to inhibit trophozoites. Hexamidine (Desomedine®), which is another diamidine typically used in the treatment of AK, was tested in this study and the results were identical to those found for hexamidine as a pure drug. This result is not surprising as Desomedine® is not the same as the Brolene® and it does not contain any preservatives in the formulation. Furthermore, the current study assessed the activity of three different diamidines with increasing alkyl chain lengths between the aromatic benzene rings. The 6-carbon hexyl chain length compound hexamidine was found to have greater activity compared with propyl and pentyl diamidines. This observation is in line with a prior study accomplished by Perrine *et al*. (1995), which reported that the anti-amoebic activity of diamidine increases with lipophilicity due to increased interaction with the lipid bilayer of *Acanthamoeba*.

3.4.4 The antimicrobial activity of biguanides

The results of the current study for chlorhexidine and PHMB are in line with earlier published studies, with PHMB exhibiting greater activity compared with chlorhexidine (Thompson *et al*., 2008, Kilvington *et al*., 2002). Typically, PHMB is used at a concentration of 0.02 % (200 µg/mL) for treating AK patients which is approximately 20 times the mean MCC for PHMB obtained from the present study. This finding agrees with the general *in vitro* sensitivities and clinical outcomes for PHMB compared to other anti-amoebic drugs (Alexander *et al*., 2015). On the other hand, an *in vitro* study undertaken by Sunada *et al*. (2014) found their *Acanthamoeba* isolates had low *in vitro* susceptibility to PHMB. There are several possible explanations for these results, firstly, the authors used a different method for cyst production which was NNA-*E. coli,* so the cysts are strong enough. Secondly, the strain types, the ATCC 300100 strain used and *Acanthamoeba* isolates obtained from patients with clinically verified AK from the Osaka University Hospital also used and the collection time for the isolates was between 1994 and 2011 and stored at 4 °C. Thirdly, the exposure times were 1 and 24 hours. Whereas in the current study we used Neff's cysts which are less resistant to the drugs, only ATCC strains used in this study which are ATCC 50370 and ATCC 30461 and the exposure time was 48 hours.

The mechanism of action of biguanides has not been fully elucidated, but PHMB and CHLX have been shown to be taken up by *Acanthamoeba* trophozoites and cysts, disrupting the cell membranes producing pentose leakage. Furthermore, there is no direct association between pentose leakage and the activity released by the biguanides (Khunkitti *et al*., 1997). The cyst stage is harder to treat than the trophozoite stage due to cysts having a thick wall which is partly made of cellulose, making it very difficult for the drugs to penetrate deep inside the wall. There is a perception that PHMB and CHLX bind to the mucopolysaccharide plug of the ostiole enabling it to pervade inside the cell membrane of the amoeba (Seal, 2003). Interestingly, this study has found that both alexidine and octenidine exhibited great *in vitro* sensitivities and indeed octenidine was better than PHMB for both species in terms of MTAC and MCC, even though toxicity against the human epithelial cell line was relatively higher. As far as we are aware, no research has assessed the activity of octenidine against cysts and trophozoites

of *Acanthamoeba*. Octenidine is commonly incorporated into the surgical hand wash Octenisan® (Schuleke and Mayer, Sheffield, U.K.). Octenidine has been formerly tested at a concentration of 1% and was found to have great sterilising activity against the tissue forms of the tapeworm *Echinococcus granulosus,* a parasite which infects some animals, including dogs and sheep, thus avoiding the transmission of infection during the eradication of a hydatid cyst (Altindis *et al*., 2004).

The findings of this study relating to alexidine are consistent with those of Alizadeh *et al*. (2009), who found amoebicidal activity at a concentration of 10 µg/mL, but they recorded a significantly higher MCC of 100 µg/mL. The specific reason for this variance is uncertain, but a possible explanation might be related to the concentration of cysts used in the above-mentioned study, which was 100-fold greater at $1 \times 10^{6,}$ compared with 1×10^{4} cells/ mL used in the current study and other published studies. At present, alexidine and octenidine are not used as treatments for AK in patients, but the antimicrobial activity of these agents against *Acanthamoeba* trophozoites and cysts, especially of octenidine, requires further investigation as they could possibly be useful in patients who do not respond to standard antiamoebic treatment. It has been reported that, when alexidine is encapsulated in one contact lens solution (AMO® RevitaLens OcuTec) at a concentration of 1.6 µg/mL, it showed great activity against *Acanthamoeba* cysts (Kilvington *et al*., 2010).

Apart from alexidine, most multi-purpose contact lens solutions (MPSs) utilise PHMB on its own or in combination with polyquaternium-1. In this study, the minimum concentration at which alexidine showed inhibitory activity against trophozoites was in the range 1 – 1.95 µg/mL, which is comparable with the concentration of PHMB contained in MPSs. Consequently, the purpose of MPSs is to provide effective disinfectant properties through the prevention and inhibition of pathogenic organisms on the contact lens or the contact lens case and, therefore, it is likely that the release of a biocide from the contact lens can exert an inhibitory effect on the viability of *Acanthamoeba* on the cornea. All types of contact lenses are exposed to the uptake and release of biocides, but the interaction is complicated and differs according to many factors. Therefore, the possible inhibitory effect on *Acanthamoeba* of the release of biocide from contact

lenses on the cornea is as yet unclear. The findings of this study showed that there is a strong relationship between *in vitro* sensitivity and TEM investigation in povidone iodine, as the damage caused to *Acanthamoeba* cysts when they are exposed to 0.5% of povidone iodine can be linked to the activity of this compound against cysts by using the microtitre method. Furthermore, the MCC value for povidone iodine was exactly the same as for PHMB and greater than for both propamidine and hexamidine. Also, the povidone iodine is not commonly used when a cornea scrape is performed, its potential inhibitory effect on the viability of *Acanthamoeba* must be taken into consideration.

A major drawback of this study is that the findings from *in vitro* sensitivities do not always correlate with clinical outcomes (Alexander *et al*., 2015). This can be explained by several factors. In the cysticidal assay, the cysts were exposed to the testing compound and then re-examined over a 7-day period for excystation and trophozoite replication. In a clinical setting, a corneal scrape for culture is performed immediately after the application of topical anaesthetics or vital stain, hence, a shorter assay period would be more representative. Furthermore, the interaction of the epithelium with a drug and the penetration of it into the stroma would be different with different types of drugs. There are limitations with *in vitro* sensitivity and efficacy studies of drugs on pathogens. Despite this, prior treatment with antibiotics to reduce pain before obtaining tissue specimens for culture or to investigate the potential adverse effects of the drugs used during clinical PCR on the viability of *Acanthamoeba* have to be considered.

3.4.5 TEM and inverted images studies

Initially, the Neff's cysts of *Acanthamoeba castellanii* (ATCC 50370) were exposed to different agents and the outcomes from this investigation led us to do more work on the same agents using TEM imaging in order to validate their effects on the Neff's cysts. Morphologically, the observations using inverted light microscopy were limited, as it can only show the cyst wall which encircles the encysted trophozoite. The other intracellular constituents, including the plasma membrane, the nucleus, the mitochondria and the lysosomes, were invisible. The TEM images presented a clear indication that BAC causes damage to the cysts. This observation is consistent with the findings of Sunada *et al*. (2014), who also found destruction of the cytoplasmic elements and a separation of the inner and

outer walls when cysts were exposed to BAC. Moreover, in the above-mentioned study, the TEM images of cyst exposed to BAC and propamidine showed damage identical to that on encysted trophozoites. This observation can be explained by the fact that the authors did not use propamidine as a pure drug but instead they tested GoldenEye® eye drops, which contain 0.1 % w/v propamidine preserved with 0.05 % benzalkonium chloride.

3.5 Conclusion

Acanthamoeba keratitis is a serious and vision-damaging infection. The present study indicates that the anti-amoebic effect of benzalkonium chloride (BAC), povidone iodine and tetracaine is greater than that of the currently used diamidines and only slightly inferior to the biguanides used as a standard treatment for AK. Also, this study showed for the first time that the octenidine hydrochloride is better than PHMB and chlorhexidine. The use of specific topical anaesthetics and ophthalmic preparations containing BAC prior to specimen sampling may affect the viability of *Acanthamoeba in vivo*, resulting in a reduced culture yield and an inhibition of the effect on PCR amplification. In order to decrease false negative culture results, ophthalmologists should be aware of the potential anti-amoebic effects of certain ophthalmic medications.

Chapter Four

Screening antimicrobials against *Acanthamoeba* spp. to identify compounds that have improved therapeutic potential for the treatment and prevention of AK

Chapter 4: Screening antimicrobials against *Acanthamoeba* spp. to identify compounds that have improved therapeutic potential for the treatment and prevention of AK

4.1 Introduction

4.1.1 *Acanthamoeba* infection and searching for better treatment

In 1973, Jones and colleagues first identified *Acanthamoeba polyphaga* keratitis as a corneal infection in south Texas, USA (Jones, 1975). A study by Stehr-Green *et al*. (1989) in the USA reported 208 cases of AK and 85% of the cases were related to the incorrect use of contact lenses. The first successful medical treatment and diagnosis of AK infection in the USA was identified in the year of 1995, and the treatment was consisting of propamidine isethionate (Brolene), neomycin sulfate-gramicidin polymyxin B sulfate (Neosporin or Ocutricin), and a 1% (v/v) to 2% (v/v) clotrimazole suspension (D'Aversa *et al*., 1995). Furthermore, recent study in the UK, exposed the contact lenses control to the domestic sinks water of bathroom and kitchens, it was found that the incidence of *Acanthamoeba* in overflow kitchen 39.1% and bathroom 25.9% (Carnt *et al.*, 2020a). Patients with *Acanthamoeba* may suffer from strong pain in their eyes, photophobia and tearing. It appears from the scientific literature that AK is worldwide problem and mainly affects contact lens wearers. This issue is further complicated by the fact that there is no licensed treatment for this infection. The similarity of symptoms between AK and other keratitis infections such as herpes simplex keratitis, leads to inaccurate diagnosis of AK (Singh and Batta, 2018), and misdiagnosis plays an important role in the progression of this infection to its advanced stages.

A range of compounds have been screened in this chapter, including aspirin analogues that tested against trophozoite and cyst, due *Acanthamoeba* has cyclooxygenase enzyme and these compounds block the action of this enzyme (Lanocha-Arendarczyk *et al*., 2018). Amidoamines were tested in this study, which are derivatives of naturally occurring plant and animal fatty acids and these compounds are available as surfactants in the cosmetic and hygiene industry (Leidreiter *et al*., 1997). Alongside the existing compounds of amidoamine,

myristamidopropyl dimethylamine (MAPD) and palmitamidopropyl dimethylamine (PAPD), new related compounds including myristoleyl-amidopropyl dimethylamine (MOPD) and palmitoleyl-amidopropyl dimethylamine (POPD) have been synthesized and tested against *Acanthamoeba*. The mechanism of action for amidoamine compounds is binding of the polar head group to the plasma membrane of *Acanthamoeba* and the carbon chain of the surfactant is integrated into the lipid bilayer of *Acanthamoeba* and then producing lateral amplification of the membrane (Denyer and Stewart, 1998). In addition, a different class of other drugs involving azole, macrolide, antineoplastic, antiparasitic, antiprotozoal and quaternary ammonium compounds were assessed for their antimicrobial activity against trophozoites and cysts. There are different mechanisms of action for the examined compounds included: azoles prevent the synthesis of ergosterol in *Acanthamoeba*. The antineoplastic interacts with carrier protein systems, causing a loss of key nutrients, and finally to cell death. The antimicrobial activity for quaternary ammonium compounds depends on the alkyl chain length and the mechanism of action for these compounds is denaturation of the important cell proteins of and *Acanthamoeba*.

4.1.2 Failure of *Acanthamoeba* to respond to current drugs

*Acanthamoeb*a differentiation leads to the recurrent insufficiency of many therapeutic agents. The relationship between treatments and *Acanthamoeba* differentiation has been widely investigated and the trophozoite stage can be effectively killed compared with the cyst stage of *Acanthamoeba* (Turner *et al.*, 2000b). On the other hand, it has been suggested that the resistance of *Acanthamoeba* to drugs is related to a range of mechanisms, including alterations in cell permeability, the modification of drug- sensitive sites, the ability of a sub-population of *Acanthamoeba* to develop drug resistance (Khan, 2009b). The mature cyst (endocyst) of *Acanthamoeba* is partly made of cellulose, which is resistant to most therapeutic agents, due the cyst consists of a thick walls, so it's difficult for the drug to penetrate inside the cyst (Lakhundi *et al*., 2015). The search continues for safe and more efficient novel therapeutic agents to combat *Acanthamoeba* spp. infection. The importance of a drug in preventing from the AK infection is related to several factors including the development of the infection

and the time at which drug intervention is commenced, the immune status of the patient and the lethal dose of *Acanthamoeba* (Schuster and Visvesvara, 2004b).

4.1.3 Aim and objectives of this chapter

In recent years, there has been increased awareness of AK and it appears from past studies that numerous *in vitro* investigations have been conducted in relation to its diagnosis and treatment. There is an urgent need to identify and develop an effective, new drug for this infection. The aim of the work described in this chapter is to examine the current therapeutic agents which are used to treat AK alongside new drugs and to assess their toxicity. To establish the antimicrobial activity for these drugs in combination against trophozoites of *Acanthamoeba* and evaluate the antimicrobial kinetics. The specific objectives of this chapter are listed below:

I. Test a variety of agents including aspirin drugs, quaternary ammonium drugs, azoles and amidoamines against cysts and trophozoites of *Acanthamoeba* spp.
II. Study the cytotoxicity of different drugs on human epithelial cell line.
III. Carry out a time kill study for the novel synthesized of amidoamine alongside the existing compounds against trophozoites.
IV. Evaluate the antimicrobial activity for several drugs in their own and in combination against trophozoites of *Acanthamoeba*.

4.2 Materials and Methods

4.2.1 Preparation of organisms

The *Acanthamoeba castellanii* (ATCC 50370) and *Acanthamoeba polyphaga* (ATCC 30461) test organisms were grown and prepared according to the methods detailed in chapter 2, section 2.4–2.8.

4.2.2 *In vitro* evaluation of the drug antimicrobial activity

The drugs were selected from different classes and included compounds previously been tested against *Acanthamoeba*, fungi , protozoa, parasite and aspirin analogues which have been found to inhibit the colorectal cancer cell lines *in vitro* and also prevent the *in vivo* colorectal tumour model in mice. The aspirin analogue compounds were obtained from Dr. Iain Nicholl in the Department of Biology, Chemistry & Forensic Science, University of Wolverhampton, U.K. The novel amidoamine compounds have been synthesised by my co-supervisor Dr Daniel Keddie in the Department of Biology, Chemistry & Forensic Science, University of Wolverhampton, U.K.

Table 4-1: List of compounds, class and source of agents that tested for their antimicrobial activity in this study

Drug	Class/Use	Solubility	Supplier
Natamycin	Macrolide/ antifungal	H_2O	Sigma (Dorset, U.K.)
Voriconazole	Antifungal	H_2O	VWR (Lutterworth, U. K.)
Posaconazole	Antifungal	H_2O	Sigma (Dorset, U.K.)
Amphotericin- B	Antifungal	H_2O	GE Healthcare (Amersham, U.K.)
Fexinidazole micronized	Antiprotozoal	H_2O	Sanofi, U.K.
Benznidazole	Antiparasitic	H_2O	VWR (Lutterworth, U. K.)
Miltefosine	Antineoplastic	5 % (v/v) Ethanol	Cayman Chemical (Michigan, USA)
Hexadecylpyridinium chloride	Quaternary ammonium	H_2O	Sigma (Dorset, U.K.)
Didecyldimethylammonium chloride	Quaternary ammonium	H_2O	Sigma (Dorset, U.K.)

Table 4.1 (continued)

Benzethonium chloride	Quaternary ammonium	H_2O	Sigma (Dorset, U.K.)
Hexadecyltrimethylammonium bromide	Quaternary ammonium	H_2O	Sigma (Dorset, U.K.)
Polyquaternium-1	Quaternary ammonium	H_2O	Sigma (Dorset, U.K.)
Myristamidopropyl Dimethylamine	Amidoamine	H_2O	Synthesised by Dr Daniel Keddie
Palmitamidopropyl Dimethylamine	Amidoamine	H_2O	Synthesised by Dr Daniel Keddie
Myristoleyl-amidopropyl dimethylamine	Amidoamine	H_2O	Synthesised by Dr Daniel Keddie (novel)
Palmitoleyl-amidopropyl dimethylamine	Amidoamine	H_2O	Synthesised by Dr Daniel Keddie (novel)

Table 4-2: List of aspirin analogue compounds that tested for their antimicrobial activity in against *Acanthamoeba* spp.

Common name, Synonym	Chemical name	Solubility
Aspirin, ortho-aspirin	Acetylsalicylic acid (aspirin)	DMSO
Diaspirin, DiA	Bis-carboxyphenylsuccinate	DMSO
benzosalin	p-aminobenzoic acid (PABA)	DMSO
Fumaryldiaspirin, F-DiA	Bis-carboxyphenyl fumarate	DMSO
Not applicable	m-bromobenzoyl salicylic acid	DMSO
Not applicable	Methyl benzoyl salicylate	DMSO
Not applicable	Isopropyl m-bromobenzoylsalicylate	DMSO
meta-aspirin	3-acetoxybenzoic acid	DMSO
para-aspirin	4-acetoxybenzoic acid	DMSO
Thioaspirin, ortho-TASP	2-acetylthiobenzoic acid	DMSO
m-thioaspirin, meta-TASP	3-acetylthiobenzoic acid	DMSO
p-thioaspirin, para-TASP	4-acetylthiobenzoic acid	DMSO

DMSO = Dimethyl Sulfoxide

The compounds were diluted in the appropriate solvent as outlined in Tables 4.1 and 4.2, and then assayed to determine their antimicrobial activity against trophozoites and cysts as described in chapter 2, sections 2.11 – 2.13. In order to determine the Minimum Trophozoite Inhibitory Concentration (MTIC), the Minimum Trophozoite Amoebicidal Concentration (MTAC) and the Minimum Cysticidal Concentration (MCC) for each of the compounds. Compounds were considered to be appropriate candidates if the compound had similar or greater antimicrobial activity to the compounds that are currently used in the treatment of AK. In addition, diluent control experiments were conducted to establish their effect on trophozoites and cysts of *Acanthamoeba* (Data not shown).

4.2.3 Toxicological testing against human epithelial cell line

After determining the antimicrobial activity for all compounds against *Acanthamoeba* spp. A different *in vitro* assay performed to determine the toxicological profile for each of compounds against a human epithelial cell line. The human epithelial cell line was maintained and prepared as outline in chapter 2, section 2.16 – 2.18.

4.2.4 Composition of the contact lens base

The solution comprised of NaCl 10 mM, KCl 1.87 mM, EDTA 0.068 mM, pluronic F127 0.30 mM, $NaH_2PO_4.H_2O$ 0.15 mM and $Na_2HPO4.7H_2O$ 0.84 mM per 800 mL of distilled water, the pH was adjusted to 7.2 with 1 M sodium hydroxide (NaOH) and the final volume of solution base made up to 1000 mL.

4.2.5 Time kill assays for amidoamine compounds

The time kill experiments where conducted for the novel and existing amidoamine compounds against trophozoites of *Acanthamoeba* spp. The reduction in viable trophozoites were plotted as change in log viability for each time point compared to zero-time viability, as described in chapter 2, section 2.13.

4.2.6 Drug synergy assays

4.2.6.1 Testing individual drug against trophozoites

In order to determine the IC_{50} for a single drug against trophozoites of *Acanthamoeba castellanii* (ATCC 50370), the experiments were accomplished in 50 mL polypropylene centrifuge tubes in triplicate by prepared the desired

concentration of drugs A or B (the MTIC for drugs A or B is 50% inhibition of trophozoites compared with the controls) were prepared and placed in 45 mL of ¼ strength Ringer's solution. Subsequently, 3 different ratios were mixed as follows: 7.5 mL of drug A or B with 2.5 mL of ¼ strength Ringer's solution (75:25), 5 mL of drug A or B with 5 mL of ¼ strength Ringer's solution (50:50) and 2.5 mL of drug A or B with 7.5 mL of ¼ strength Ringer's solution (25:75). Drugs A and B were tested on their own by preparing the required concentrations of both drugs in 10 mL of ¼ strength Ringer's solution and also a positive control was run in a ¼ strength Ringer's solution. Then, 100 μL of 5 x 10^6 cells/mL of trophozoites were added to each tube and the tubes were placed at room temperature for 24 hours. After that, the tubes were plated out using a time kill assay as described in chapter 2, section 2.14.

4.2.6.2 Synergy testing for combinations of two drugs against trophozoites

These experiments were designed to assess the antimicrobial activity of the drugs in combination against trophozoites of *A. castellanii* (ATCC 50370) as an alternative to single drug testing. The experiments were performed in triplicate in 50 mL polypropylene centrifuge tubes by adding the desired concentration of drug A or B (the MTIC for drug A or B is 50% inhibition of trophozoites compared with the controls) to a 45 mL of ¼ strength Ringer's solution. The tubes were labelled as drug A and drug B, with tubes 1 and 5 containing drug A and B respectively. The other 3 tubes contained combinations of drugs A and B in different ratios: 7.5 mL of drug A mixed with 2.5 mL of drug B (75:25), 5 mL of drug A mixed with 5 mL of drug B (50:50) and 2.5 mL of drug A mixed with 7.5 mL of drug B (25:75). Tube 6 serves as a positive control and contained a ¼ strength Ringer's solution. 100 μL of 5 x 10^6 cells/mL of trophozoites were added to each tube and the tubes were placed at room temperature for 24 hours. Then the tubes were plated out using a time kill assay as described in chapter 2, section 2.14.

4.2.6.3 Data analysis for single drug and the combinations of two drugs

Initially, the concentrations of the drugs were selected from MTIC study of trophozoites of *A. castellanii* (ATCC 50370). The log kill caused by drug A or B alone against trophozoites from the time kill experiments was expressed as a percentage in Microsoft Excel against the drug concentrations. If no kill was observed, that indicated a 0% log kill of trophozoites, 1-log kill equalled 25%, 2-log kill was expressed as 50%, 3-log kill was 80% and 4-log kill was 99%. The IC_{50} for drug A or B in their own was determined by plotting a semi-log against the log kill of trophozoites. Subsequently, the IC_{50} for the combined drugs was determined by drawing a log kill curve with a semi log of drug A alone and drug A combined with drug B. Then, the IC_{50} for drug A was combined with drug B and compared to the IC_{50} for drug A alone. The same process was repeated for drug B.

The fractional inhibitory concentration (ΣFIC) was calculated according to the formula below:

ΣFIC = FIC (Drug A) + FIC (Drug B)

$$\text{FIC (Drug A)} = \frac{IC_{50}\ \text{Drug A (in presence of B)}}{IC_{50}\ \text{Drug A (alone)}}$$

$$\text{FIC (Drug B)} = \frac{IC_{50}\ \text{Drug B (in presence of A)}}{IC_{50}\ \text{Drug B (alone)}}$$

The interaction between the assessed drugs was determined from the ΣFIC value:

If the ΣFIC ≤ 0.5 indicates synergistic effect; If the ΣFIC >0.5 but ≥4 indicates additive effect; and if the ΣFIC >4 indicates antagonistic effect (Odds, 2003).

4.3 Results

This section is presenting the antimicrobial testing data for different group of drugs against both trophozoites/cysts and the toxicity for human epithelial cell line, the time kill for novel and existing amidoamine compounds and the findings for single drug and in combination against trophozoites of *Acanthamoeba castellanii* (ATCC 50370).

4.3.1 Novel and existing of amidoamines

The C14 myristamidopropyl dimethylamine (MAPD) showed huge activity against cysts ranged from 7.8 – 15.6 µg/mL and against trophozoites between 1 – 3.9 µg/mL. The toxicity test for human cell line comparable to that obtained against cysts at MCT 7.8 µg/mL. The next novel compound evaluated was a C14-1 myristoleyl-amidopropyl-dimethylamine (MOPD), which showed a minor antimicrobial activity against cysts between 125 – 250 µg/mL and against trophozoites ranged from 31.3 – 125 µg/mL for both species, also this compound was not toxic human epithelial cell line at MCT 15.6 µg/mL. Another compound was tested a C16 palmitamidopropyl dimethlamine (PAPD) and was found to be active against the trophozoites between 3.9 – 15.6 µg/mL and against cysts ranged from 15.6 – 31.3 µg/mL for either species. Toxicity to human epithelial cell line was same as MCC values. The C16-1 palmitoleyl-amidopropyl-dimethylamine (POPD) was found less active against cysts and had activity between 31.3 – 62.5 µg/mL and was found to be more active against trophozoites at 3.9 – 31.3 µg/mL (Table 4.3).

Table 4-3: Efficacy of amidoamine novel and exciting compounds against trophozoites and cysts for *A. polyphaga* & A. *castellanii* and for their toxicity against a human epithelial cell line (Hep2)

			In vitro drug sensitivities (µg/mL)							
			A. castellanii (ATCC 50370)			*A. polyphaga* (ATCC 30461)			Hep2 cells	
Chemical Name	Abbreviation	Hydrophobic Chain length: number of alkenes	MTIC*	MTAC**	MCC***	MTIC*	MTAC**	MCC***	MIC+	MCT++
Myristamidopropyl Dimethylamine	MAPD	C14	1	3.9	7.8	1.95	3.9	15.6	3.9	7.8
Palmitamidopropyl Dimethylamine	PAPD	C16	7.8	15.6	15.6	3.9	7.8	31.3	15.6	31.3
Myristoleyl-amidopropyl dimethylamine	MOPD	C14:1	62.5	125	250	31.3	62.5	125	7.8	15.6
Palmitoleyl-amidopropyl dimethylamine	POPD	C16:1	15.6	31.3	62.5	3.9	7.8	31.3	15.6	31.3

MTIC* Minimum trophozoite inhibitory concentration, MTAC** Minimum trophozoite amoebicidal concentration, MCC*** Minimum cysticidal concentration, MIC+ Minimum inhibitory concentration, MCT++ Minimum Cytotoxic Concentration. It is clear from the results presented in Table 4-3 that the MAPD of amidoamine compounds demonstrated higher antimicrobial activity against trophozoites and cysts of *Acanthamoeba* relative to the other compounds evaluated.

4.3.2 Compounds that showed favourable antimicrobial activity against cysts and trophozoites of *Acanthamoeba* spp.

A series of different class of drugs have been shown encouraging activity against both trophozoite and cyst. Posaconazole was active against cysts within the concentration range 7.8 – 15.6 µg/mL and more activity was observed against trophozoites at concentrations of between 1 and 7.8 µg/mL. However, it was toxic for the human epithelial cell line at MCT 3.9 µg/mL. Voriconazole was inactive against cysts of both species of *Acanthamoeba* at an MCC of 500 µg/mL, amoebicidal activity occurred at a concentration of 31.3 µg/mL and inhibitory activity at concentrations between 7.8 and 3.9 µg/mL. When it was tested against the human epithelial cell line, it showed level of toxicity at MCT 7.8 µg/mL. Amphotericin-B showed no activity against cysts at MCC >500 µg/mL and also showed no amoebicidal activity for either species of *Acanthamoeba* at MTAC 500 µg/mL. However, it was tolerated by the human epithelial cell line within the range 62.5 – 125 µg/mL. Natamycin is a macrolide and showed no activity against cysts at MCC >500 µg/ml but was active against trophozoites within the range 1.95 – 3.9 µg/mL for the two species of *Acanthamoeba*. It was toxic for the human epithelial cell line between 3.9 and 7.8 µg/mL (Table 4.4).

Miltefosine is antineoplastic, it was very active against trophozoites within the concentration range 0.5 –1.95 µg/mL and it was also active against cysts in the MCC range of 31.3 – 62.5 µg/mL. This compound was slightly toxic for the human epithelial cell line at MCT 15.6 µg/mL. The antiprotozoal compound tested was fexinidazole micronized. It was found to be inactive against cysts at an MCC of 500 µg/mL but showed moderate activity against trophozoites with a MTAC range of 15.6 – 62.5 µg/mL. Fexinidazole was toxic to the human cell line at MCT 250 µg/mL. The antiparasitic drug benznidazole was found to have no cysticidal activity at MCC 500 µg/mL, however this compound was found to have acceptable amoebicidal activity within the range 31.3 – 62.5 µg/mL. It showed no toxicity against human epithelial cell line at MCT 31.3 µg/mL (Table 4.4).

For quaternary ammonium drugs, didecyldimethylammonium chloride showed incredible activity against cysts at MCC ranging from 3.9 – 7.8 µg/mL. It was very active against trophozoites and it showed inhibitory activity at a concentration of 0.48 µg/mL. This compound demonstrated amoebicidal activity at concentrations between 0.97 and 1.95 µg/mL. Hexadecyltrimethylammonium bromide was active against cysts at MCC 7.8 – 15.6 µg/mL and showed great activity against trophozoites between 1.95 and 7.8 µg/mL. Polyquaternium-1 showed massive inhibitory activity against trophozoites at concentrations between 1.95 and 3.9 µg/mL. Amoebicidal activity occurred within the range 3.9 – 7.8 µg/mL. This compound was active against *Acanthamoeba* cysts for both species at MCC ranging from 15.6 – 31.3 µg/mL. Toxicity for the human epithelial cell line occurred at the same concentration as cysticidal activity for *A. polyphaga* at MCT 15.6 µg/mL (Table 4.4). Benzethonium chloride was active against trophozoites at concentrations between 3.9 and 15.6 µg/mL and exhibited moderate activity against cysts between 13.3 – 62.5 µg/ml. It was toxic for the human cell line at MCT 7.8 µg/mL. Unlike the other quaternary ammonium drugs which were tested, hexadecylpyridinium chloride produced only limited activity against trophozoites within the range 62.5 – 250 µg/mL and it was inactive against *Acanthamoeba* cysts for either species at MCC 500 µg/ml. The MCT for this compound was found to be at 62.5 µg/mL (Table 4.4).

Table 4-4: Efficacy of different agents that showed great antimicrobial activity against trophozoites and cysts of *Acanthamoeba* spp. and the toxicity against human epithelial cell line.

	In vitro drug sensitivities (µg/mL)							
	A. castellanii (ATCC 50370)			*A. polyphaga* (ATCC 30461)			Hep2 cells	
Drug	MTIC*	MTAC**	MCC***	MTIC*	MTAC**	MCC***	MIC+	MCT++
Natamycin	1.95	3.9	>500	1.95	3.9	>500	3.9	7.8
Voriconazole	7.8	31.3	500	3.9	31.3	500	3.9	7.8
Posaconazole	1	3.9	7.8	1	7.8	15.6	1.95	3.9
Amphotericin- B	250	500	>500	500	500	>500	62.5	125
Fexinidazole micronized	15.6	62.5	500	7.8	15.6	500	125	250
Benznidazole	7.8	31.3	500	7.8	62.5	500	15.6	31.3
Miltefosine	1	1.95	62.5	0.5	1	31.3	7.8	15.6
Benzethonium Chloride	3.9	7.8	31.3	7.8	15.6	62.5	15.6	7.8
Hexadecylpyridinium Chloride	62.3	125	500	125	250	500	125	62.3
Didecyldimethylammonium chloride	0.48	0.97	3.9	0.48	1.95	7.8	31.3	15.6
Hexadecyltrimethylammonium bromide	1.95	3.9	7.8	3.9	7.8	15.6	62.5	31.3
Polyquaternium-1	3.9	7.8	31.3	1.95	3.9	15.6	31.3	15.6

MTIC* Minimum trophozoite inhibitory concentration, MTAC** Minimum trophozoite amoebicidal concentration, MCC*** Minimum cysticidal concentration, MIC+ Minimum inhibitory concentration, MCT++ Minimum Cytotoxic Concentration. The most active compounds against trophozoites and cysts from the data that presented in this table are miltefosine, posaconazole and the quaternary ammonium compounds in particular didecyldimethylammonium chloride.

4.3.3 The activity of aspirin compounds against cysts and trophozoites of *Acanthamoeba* spp.

Aspirin analogue compounds showed minor antimicrobial activity against cysts for both species of *Acanthamoeba* and the MCC values ranged between 250 – 500 µg/mL. As well as these compounds were not toxic against human epithelial cell line and more tolerated between 62.5 – 500 µg/mL. When these compounds assessed for their antimicrobial activity against trophozoites, it was the amoebicidal activity between 15.6 – 125 µg/mL as range and the trophozoites inhibitory was ranged from 7.8 – 62.5 µg/mL for either species of *Acanthamoeba*. Among all the aspirin analogue compounds tested against cysts of *Acanthamoeba* in this study there were only two compounds, namely bis-carboxyphenyl fumarate and isopropyl *m*-bromobenzoyl salicylate, active against cysts between 15.6 – 62.5 µg/mL and the amoebicidal activity was at range of 7.8 – 31.3 µg/mL for both species of *Acanthamoeba* (Table 4.5).

Table 4-5: Efficacy of aspirin analogues drugs against trophozoites and cysts of *Acanthamoeba* spp., and the toxicity against human epithelial cell line.

	In vitro drug sensitivities (ug/mL)							
	A. castellanii (ATCC 50370)			*A. polyphaga* (ATCC 30461)			Hep2 cells	
Chemical name	MTIC*	MTAC**	MCC***	MTIC*	MTAC**	MCC***	MIC+	MCT++
acetylsalicylic acid (aspirin)	15.6	62.5	500	7.8	15.6	500	125	250
Bis-carboxyphenylsuccinate	15.6	31.3	250	7.8	31.3	250	62.5	125
p-aminobenzoic acid	31.3	125	500	7.8	15.6	500	62.5	125
Bis-carboxyphenyl fumarate	3.9	7.8	15.6	7.8	15.6	31.3	62.3	125
m-bromobenzoyl salicylic acid	31.3	125	500	7.8	31.3	250	125	250
Methyl benzoyl salicylate	31.3	62.5	500	7.8	31.3	500	125	250
Isopropyl m-bromobenzoyl salicylate	7.8	15.6	31.3	7.8	31.3	62.5	250	500
3-acetoxybenzoic acid	62.5	125	500	15.6	62.5	500	125	250
4-acetoxybenzoic acid	31.3	125	500	15.6	62.5	500	125	250
2-acetylthiobenzoic acid	31.3	62.5	500	15.6	62.5	250	250	500
3-acetylthiobenzoic acid	62.5	125	500	31.3	62.5	500	62.5	125
4-acetylthiobenzoic acid	31.3	62.5	500	31.3	62.5	500	125	250

MTIC* Minimum trophozoite inhibitory concentration, MTAC** Minimum trophozoite amoebicidal concentration, MCC*** Minimum cysticidal concentration, MIC+ Minimum inhibitory concentration, MCT++ Minimum Cytotoxic Concentration. There are only two compounds included bis-carboxyphenyl fumarate and isopropyl m-bromobenzoyl salicylate that showed good activity against both trophozoites and cysts of *Acanthamoeba*. Also, the same tested compounds demonstrated no toxicity towards the human epithelial cell line.

4.3.4 Time kill studies for amidoamines

More investigations for the novel compounds (including myristoleyl-amidopropyl dimethylamine MOPD and palmitoleyl-amidopropyl dimethylamine POPD) and existing amidoamines (involving myristamidopropyl dimethylamine MAPD and palmitamidopropyl dimethylamine PAPD) were made to establish the antimicrobial activity of these compounds over the time. When they were formulated into a contact lens base solution at a concentration of 0.0005 % (w/v) and tested against trophozoites of *Acanthamoeba,* they were found to be more active as shown in Figures 4.1 and 4.2.

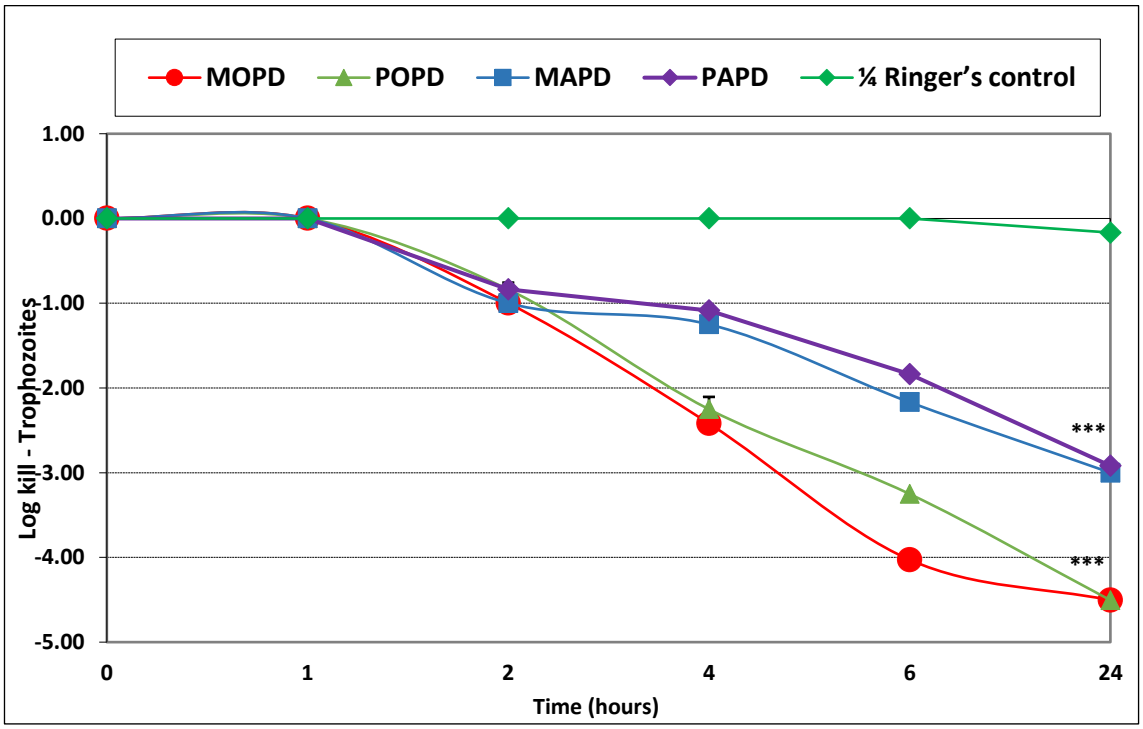

Figure 4-1: Efficacy of novel amidoamines including myristoleyl-amidopropyl dimethylamine (MOPD), palmitoleyl-amidopropyl dimethylamine (POPD), compared to the current compounds of amidoamines; myristamidopropyl dimethylamine (MAPD) and palmitamidopropyl dimethylamine (PAPD) at concentration (0.0005% w/v) formulated in contact lens base solution and tested against trophozoites of *Acanthamoeba castellanii* (ATCC 50370). One-Way Analysis of Variance (ANOVA) was performed. Asterisks represent values statistically significant (***P<0.001) between the tested compounds of amidoamine and the ¼ strength Ringer's control.

Interestingly, MOPD and POPD showed higher antimicrobial activity against trophozoites, as they gave complete kill at a 4.5 log reduction after 24 hours. However, the activity against trophozoites was lower at 3-log reduction for MAPD and PAPD with the same time exposure. One-Way ANOVA analysis confirmed that MOPD and POPD were significant ($P < 0.001$) in their antimicrobial activity after 24 hours compared to a ¼ strength Ringer's control and there was also a significant difference ($P < 0.001$) for MOPD and POPD after 24 hours in terms of their activity against trophozoites compared with MAPD and PAPD (Figure 4.1). Furthermore, two sample t-test for the means showed that a significance correlation between the tested compounds after 24 hours-time point and a ¼ strength Ringer's control, as the p value was observed at p=0.018

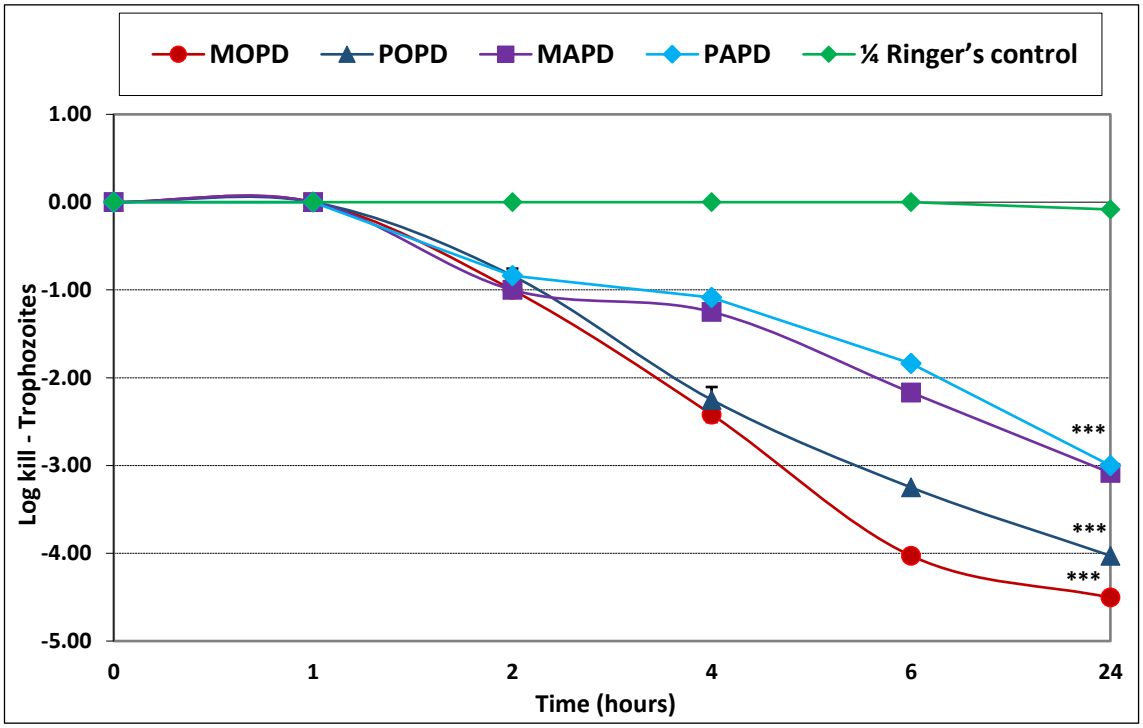

Figure 4-2: Efficacy of novel amidoamines including myristoleyl-amidopropyl dimethylamine (MOPD), palmitoleyl-amidopropyl dimethylamine (POPD), compared to the current compounds of amidoamines; myristamidopropyl dimethylamine (MAPD) and palmitamidopropyl dimethylamine (PAPD) at concentration (0.0005% w/v) formulated in contact lens base solution and tested against trophozoites of *Acanthamoeba polyphaga*(ATCC 30461). One-Way Analysis of Variance (ANOVA) was performed. Asterisks represent values statistically significant (***P<0.001) between the tested compounds of amidoamine and the ¼ strength Ringer's control.

MOPD achieved complete kill against trophozoites of *A. polyphaga* (ATCC 30461) and provided a 4.5 log reduction at 24 hours. The rate of kill slightly declined to a 4-log reduction for POPD at the same time point. Moreover, the level of the kill was only a 3-log reduction for both MAPD and PAPD against trophozoites of the same tested strain after 24 hours of exposure. There was a significant difference ($P < 0.001$) after 24 hours with regards of the activity against trophozoites between MOPD, POPD compared with a ¼ strength Ringer's control and also in comparison with MAPD and PAPD over 24 hours of time point (Figure 4.2). In addition, two sample t-test for the means demonstrated that a significance differences between the assessed compounds over 24 hours and the ¼ strength Ringer's control as the p value was recorded at $p=0.009$.

4.3.5 Single and combination of two drugs findings

Table 4.6 below presents the IC_{50} findings for a number of drugs as individual and in combination against trophozoite of *Acanthamoeba castellanii* (ATCC 50370). The results in (Table 4.7) are comparing the activity of two drugs against trophozoites of *A. castellanii* (ATCC 50370). Amphotericin-B and chlorhexidine (CHLX) as individual drugs were tested at a range of concentrations from 31.3 to 250 µg/mL for amphotericin-B and from 3.9 to 15.6 µg/mL for CHLX. These concentrations were plotted as logs in Microsoft Excel. The same concentrations were used in combination assays. The IC_{50} for amphotericin-B was recorded at higher value of 73.6 µg/mL, whereas the IC_{50} for CHLX was observed at 5.64 µg/mL. When the CHLX was combined with amphotericin-B, the IC50 was dropped to 3.23 µg/mL and this finding suggested that the CHLX has a positive effect when it combined with amphotericin-B. The fractional inhibitory concentration (ΣFIC) for both drugs was found to be 1.14 which indicates there was an additive effect from the combination of these drugs.

Voriconazole and CHLX were tested at concentrations of 3.9 – 31.3 µg/mL and 3.9 –15.6 µg/mL respectively. As single drug of voriconazole gave an IC_{50} of 16.53 µg/mL in comparison with the IC_{50} which obtained from CHLX alone at 5.64 µg/mL. However, much lower value of IC_{50} was found at 6.87 µg/mL for the combination of these drugs. Furthermore, the outcome of the combination study showed that the ΣFIC value for the two drugs was 2.4 and that resulted in the additive effect. The susceptibility of *A. castellanii* trophozoites to CHLX and

pentamidine (aromatic diamidine) was investigated. The selected concentrations were between 7.8 and 62.3 µg/mL for pentamidine and between 3.9 and 15.6 µg/mL for CHLX. The IC_{50} for pentamidine was detected at 59.9 µg/mL compared with only 5.64 µg/mL for CHLX as individual drug. The combination of CHLX with pentamidine was led to the IC_{50} of 11.98 µg/mL and this result proposed that the CHLX had a positive impact when it mixed with pentamidine and tested against trophozoites. This study found that there is an additive effect from the combination of these drugs as the ΣFIC value was 1.98. The effects on *A. castellanii* trophozoites of CHLX and natamycin, both individually and in combination, were examined. The selected concentrations of natamycin and CHLX were 1.95 –15.6 µg/mL and 3.9 –15.6 µg/mL respectively. The IC_{50} results achieved from this study for natamycin at 7.82 µg/mL and a lower IC_{50} value of CHLX of 5.64 µg/mL was recorded.

These findings indicated that the antimicrobial activity of natamycin was enhanced in the presence of CHLX. It was established that the ΣFIC for the combination of these drugs was 1.8 and this value suggested there was an additive effect. One Way ANOVA statistical analysis showed a significant correlation ($P < 0.001$) for CHLX concentrations compared to all drugs that tested in this study. Also, using two sample t-test for comparing the means showed that a significance differences between the IC50 for the examined compounds as individual or in combination as the p value was found to be at p=0.04431.

Table 4-6: Efficacy of single drugs against trophozoites of *A. castellanii* (ATCC 50370).

Single drug	IC_{50}	Combinations	IC_{50}
Chlorhexidine	5.64 ± 0.02	Chlorhexidine + Amphotericin-B	3.23 ± 0.99
Amphotericin-B	73.6 ± 1.18	Chlorhexidine + Natamycin	3.94 ± 0.98
Natamycin	7.72 ± 1.02	Chlorhexidine + Pentamidine	11.98 ± 1.05
Pentamidine	59.9 ± 1.11	Chlorhexidine + Voriconazole	6.87 ± 1.01
Voriconazole	16.53 ± 1.05		

The results expressed as the mean of three independent experiments ± standard deviation (SD) after 24 hours exposure time. One-Way Analysis of Variance (ANOVA) was utilized for these experiments.

Table 4-7: Efficacy of drug combinations against trophozoites of A. castellanii (ATCC 50370).

Combinations	ΣFIC	Relationship
Chlorhexidine + Amphotericin-B	1.14	Additive
Chlorhexidine + Natamycin	1.8	Additive
Chlorhexidine + Pentamidine	1.98	Additive
Chlorhexidine + Voriconazole	2.4	Additive

4.4 Discussion

This section provides a comprehensive discussion about the mechanism of action for a number of drugs including antifungal, macrolide, antineoplastic, antiprotozoal, quaternary ammonium, aspirin drugs and amidoamines. The time kill for novel and existing compounds of amidoamines and also the combination of two drugs against trophozoites.

4.4.1 Antifungal/macrolide/antineoplastic drugs

In this study, several of triazole antifungal agents including voriconazole, posaconazole, amphotericin-B and natamycin have been tested against trophozoites and cysts. The chemical structures for these compounds are different as can be seen in Figure 4.3. The mechanism of action for the antifungals is block the synthesis of ergosterol in *Acanthamoeba* and lead to increased permeability and cell ion leakage. Miltefosine (antineoplastic) drug has also been tested and the chemical structure for this drug consists of hexadecyl phosphocholine monoester (Figure 4.3.E).

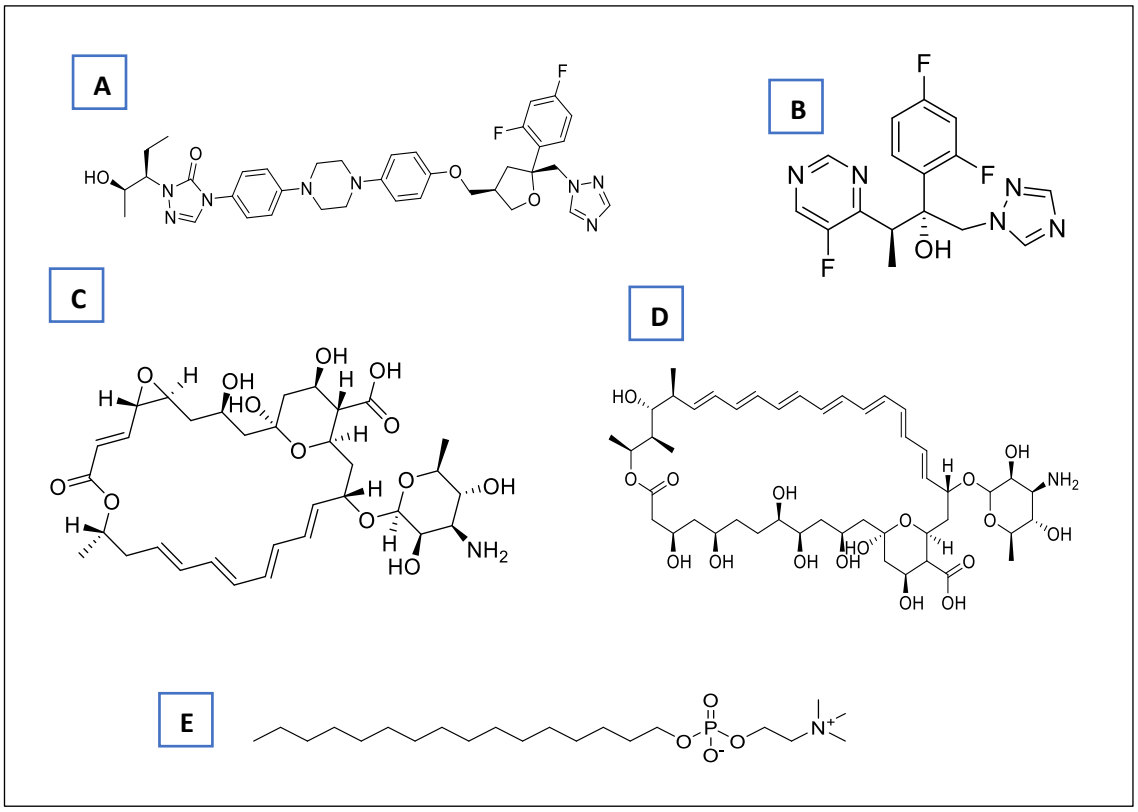

Figure 4-3: Chemical structure of (A) Posaconazole (B) Voriconazole, (C) Natamycin, (D) Amphotericin- B, (E) Miltefosine.

The first compound tested was voriconazole, which showed no activity against cysts at a concentration of MCC 500 µg/mL, but it was highly active against trophozoites. It inhibited trophozoite growth at concentrations of between 3.9 – 7.8 µg/mL and showed amoebicidal activity at 31.3 µg/mL. The results obtained in this study reflect a previous study (Schuster *et al*., 2006) in which activity against trophozoites at 40 µM and this concentration is similar to 16 µg/mL was observed but with no cysticidal activity. However, the authors utilized a different methodology to that study, as they tested a clinical strain with a final concentration of $1x10^6$ cells/mL and the time for exposure was 10 days, whereas in the current study the final concentration of cell density for cysts or trophozoites was 1×10^4 cells/mL and the exposure time for trophozoites and cysts was 1 and 2 days respectively. The findings of the present study contradict the outcomes of past study by Iovieno *et al*. (2014b) who found that, for voriconazole, the MCC at which observed activity occurred against the ATCC strain and clinical isolates was 33.13 and 46.25 µg/mL respectively. Voriconazole had previously been shown to have higher activity against *Acanthamoeba* cysts with IC_{50} of 4.59 µg/mL (IC_{50} means 50% inhibition of trophozoites) and IC_{90} at concentration of 15.43 µg/mL (IC_{90} indicates 90% inhibition has occurred for trophozoites) (Martin-Navarro *et al*., 2013). The observed increased in the activity of voriconazole against cyst of *Acanthamoeba* could be related to the drug version used by the authors, they tested eye drops (VFEND®) of voriconazole which are preserved with sulfobutyl ether β-cyclodextrin sodium as this salt may behind the activity and also the incubation period for around 1 week. In contrast, the current study tested the voriconazole as a pure drug with less exposure time of 3 days.

Furthermore, another possible explanation for the significant differences between these previous studies and the present study is that the studies by (Martín-Navarro *et al*., 2013, Iovieno *et al.*, 2014b) used IC_{50}, IC_{90}, MMC_{50} and MMC_{90} to evaluate cysticidal activity whereas in the current study, only the activity for total kill for a given concentration is reported. Posaconazole was found to be active against *Acanthamoeba* cysts at MCC concentrations ranging from 7.8 – 15.6 µg/mL. This compound showed great activity against trophozoites, with inhibition of 1 µg/mL for both species of *Acanthamoeba* and amoebicidal concentrations ranged from 3.9 – 7.8 µg/mL. In this study, the MCC of posaconazole was

observed between 7.8 – 15.6 µg/mL and these values are lower than that seen in the study by (Iovieno et al., 2014b) which reported 43.75 µg/mL for clinical isolates and was higher up to 52.5 µg/mL for ATCC isolates. The posaconazole had greater clinical efficacy than voriconazole against fungal keratitis (Torres *et al.*, 2005). A recent *in vitro* research reported high effective concentration (EC_{50}) activity of posaconazole and voriconazole at 0.045 µM which equal to (0.031 µg/mL) and 0.6 µM which equivalent to (0.2 µg/mL) respectively against trophozoites of *Acanthamoeba castellanii* clinical strains (Shing *et al*., 2020) and these findings are consistent with those obtained for both drugs against trophozoites in the current study

The antineoplastic agent miltefosine had previously been shown to have great activity against *Leishmania donovani* (Achterberg, 1987), and also against *Trypanosoma cruzi,* which causes Chagas disease (Saraiva *et al*., 2002). The mechanism by which miltefosine acts is to interfere with carrier proteins systems, leading to a reduction in essential nutrients, and eventually to cell death (Croft *et al*., 2003). In the present study, miltefosine showed excellent activity against trophozoites of both species in the range 0.5 – 1.95 µg/mL. However, it was less active against cysts with an MCC of 31.3 – 62.5 µg/mL. It is interesting to note that the miltefosine showed slight toxicity against the human cell line at MCT 31.3 µg/mL. The findings from the present study largely support those of (Walochnik *et al*., 2002), who tested miltefosine along with alkiphosphocholines against three different strains of *Acanthamoeba,* including *A. castellanii* (strain 1BU), *A. polyphaga* (strain 5SU) and A*. lenticulata* (strain 72/2). In Walochnik *et al*. (2002) study, time kill was performed in order to evaluate antimicrobial activity of miltefosine over time. Great activity was found against trophozoites of all three strains of *Acanthamoeba* at <30 min at a concentration of 80 µM which is equivalent to the 32 µg/mL. When the same testing was repeated with cysts from the three different strains, miltefosine showed a 3 – 4 log reduction over 24 hours.

A more recent *in vitro* investigation conducted by Chao *et al*. (2020) which tested the miltefosine at the concentration of 2.42, 4.84, 9.68, 19.36, 38.72 and 77.44 mM for different times: 1, 3, 5, 7 days at 37 °C against cysts of *Acanthamoeba* species associated to the genotypes T3, T4 and T5. All concentrations assessed of miltefosine showed that a 100% elimination of cyst, but the level of activity was different against several genotypes, the miltefosine at a concentration of 38.72 mM killed both T4 and T5 after 1 day of incubation, whereas the killing of T3 required a higher concentration at 77.44 mM with similar incubation time (Chao *et al*., 2020). In a newer case study, miltefosine was used as an effective oral treatment for patients who had a microbial diagnosis of *Acanthamoeba*, and the findings of this research showed that two patients improved after only 1 week of miltefosine treatment (Naranjo *et al*., 2020). The antifungal macrolide natamycin showed reasonable activity against trophozoites of both species but was not active against cysts at an MCC >500 µg/mL. This result is in line with *in vitro* study performed by Nakaminami *et al*. (2017) which found a higher activity for natamycin against trophozoites after 7-day with IC_{90} of 4.1 µg/mL and after 12 hours the IC_{90} found at 11.6 µg/mL. Amphotericin- B showed no activity against cysts of both species of *Acanthamoeba* at MCC >500 µg/mL and it was also inactive against trophozoites. The results obtained from this study support early medical treatments by Wright *et al*. (1985), who confirmed that amoebicidal activity for amphotericin-B was >125 µg/mL.

4.4.2 Antiparasitic/ antiprotozoal drugs

Benznidazole, an antiparasitic made of carboxy group of (2-nitroimidazol-1-yl)acetic acid and aromatic amino group of benzylamine (Figure 4.4.A). This drug exhibited elevated activity against trophozoites, but when the experiment was repeated against cysts, it showed no activity for either species of *Acanthamoeba* with MCC >500 µg/mL. Benznidazole had previously been shown to have higher activity against the protozoan parasitic *Trypanosoma cruzi* in mouse models (Garcia *et al*., 2005). Fexinidazole micronized ((1H-imidazole, 1-methyl-2-((4-(methylthio)phenoxy) methyl)-5-nitroimidazole) (Figure 4.4.B) is an antiprotozoal and exhibited activity against trophozoite between 7.8 up to 62.5 µg/mL and the activity against cyst was found at a higher concentration of 500 µg/mL.

Previously, the fexinidazole had an MIC of 5 µg/mL against *Trypanosoma cruzi* (Raether and Seidenath, 1983).

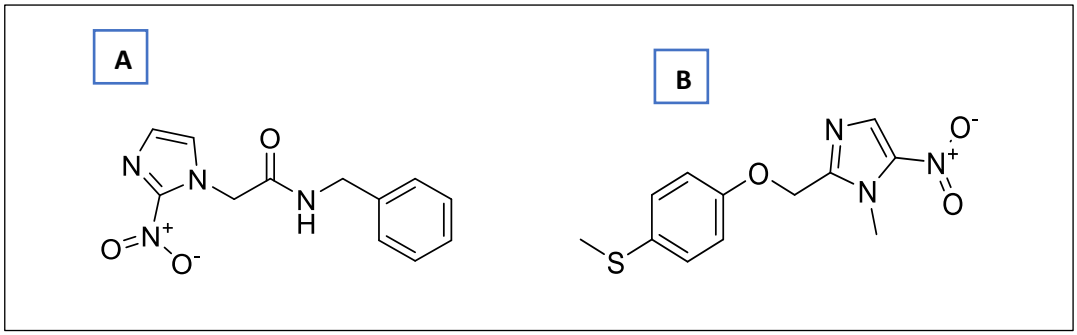

Figure 4-4: Chemical structure of (A) Benznidazole, (B) Fexinidazole micronized.

4.4.3 Quaternary ammonium compounds

Among the quaternary ammonium compounds tested in this study, didecyldimethylammonium chloride which has 2 long alkyl groups (Figure 4.5.A) demonstrated excellent antimicrobial activity against trophozoites and cysts of both species of *Acanthamoeba* at lowest concentrations and as far as we know, this compound has not been tested against *Acanthamoeba* before. The chemical structures for quaternary ammonium compounds consist of no less than one hydrophobic hydrocarbon chain that is attached to a positively charged nitrogen atom and other alkyl groups (Figure 4.5). It is believed that the antimicrobial activity of these compounds is related to the alkyl chain length (McBain *et al.*, 2004). The results obtained of this study reflect the findings of a previous study by Beier *et al*. (2015), which found that the didecyldimethylammonium chloride very active against the gram-negative bacteria *Pseudomonas aeruginosa* at MIC_{50} of 16 µg/mL and MIC_{90} of 32 µg/mL. Didecyldimethylammonium chloride has been used across a broad spectrum against different types of bacteria, including *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* (Walsh *et al*., 2003). Additionally, the mechanisms by which these compounds act are related to denaturation of the key cell proteins and disruption of the cell membrane (Gerba, 2015). The present findings for benzethonium chloride also support (Yip *et al*., 2006) study which found antimicrobial activity for this compound against cancer cell lines at 3.8 µmol/L. However, it contradicts the findings of Lukáč *et al*. (2013) which reported that the minimal trophocidal concentration (MTC) for this compound was 250 µM which is equivalent to 112

µg/mL against *Acanthamoeba lugdunensis*, and a double MTC value of 500 µM comparable with 224 µg/mL was observed for *Acanthamoeba quina*.

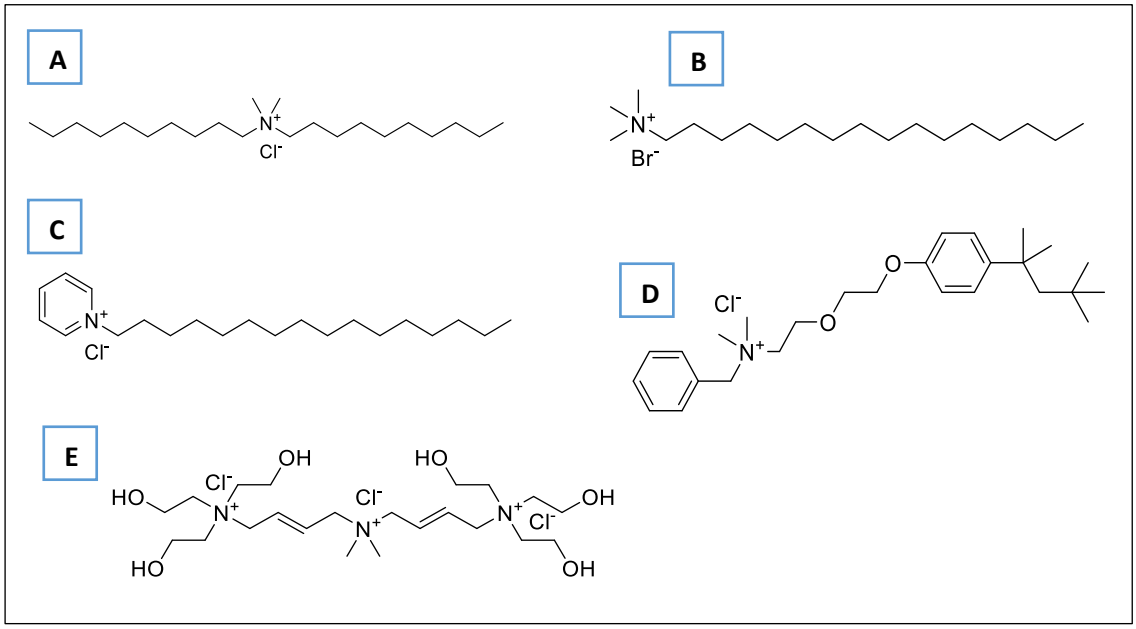

Figure 4-5: The chemical structures of (A) Didecyldimethylammonium chloride (B) Hexadecyltrimethylammonium bromide, (C) Hexadecylpyridinium Chloride, (D) Benzethonium Chloride , (E) Polyquaternium-1

In this study, hexadecyltrimethylammonium bromide produced elevated activity against trophozoites and *Acanthamoeba* cysts at lowest concentrations and the results are in line with the past study by Lukac *et al*. (2013), which found this compound is active against *Candida albicans* and *Acanthamoeba lugdunensis* at 15.6 µM which is equal to 5.6 µg/mL. An *in vitro* study by Zanetti *et al.* (1995) reported that polyquaternium-1 had no activity when incorporated into a contact lens solution and tested over time against *Acanthamoeba* trophozoites and cysts. This finding was at variance with the results of the current study. The only quaternary ammonium drug tested which showed antimicrobial activity at highest concentrations was hexadecylpyridinium chloride. This provides support for a pervious study by Beier *et al*. (2015) which tested this compound against *Pseudomonas aeruginosa* and MIC_{50} and MIC_{90} obtained were 128 and up to 256 µg/mL respectively.

4.4.4 Aspirin analogues

In the current study, several aspirin analogues are tested for the first time against cysts and trophozoites of *Acanthamoeba*, and the results have shown that the minimum cysticidal concentration values range from 15 µg/mL to 500 µg/mL. The minimum trophozoite inhibitory concentration ranged from 3.9 µg/mL to 62.5 µg/mL and the minimum trophozoite amoebicidal were observed at between 15.6 –125 µg/mL. Aspirin, also commonly known as acetylsalicylic acid, is classified as a member of nonsteroidal anti-inflammatory drugs (NSAIDs) (Tosco and Lazzarato, 2009). The aspirin-related compounds tested in the present study, previously have been shown a huge activity against cell proliferation and caused apoptosis of colorectal cancer cells (Deb *et al*., 2011, Claudius *et al*., 2014). The mechanism of action for aspirin analogues is inhibit the activity of the cyclooxygenase (COX) enzyme in cancer cell line (Zha *et al*., 2004). The function for COX in human is synthesising of prostaglandins as a response to pain signals which produce inflammation in the body (Vane and Botting, 2003).

As revealed in subheading 3.1.1 of this chapter, the COX enzyme exists in *Acanthamoeba* and so aspirin analogues should inhibiting the activity of this enzyme (Lanocha-Arendarczyk *et al*., 2018). In an *in vivo* study, it was reported that *Acanthamoeba* produced a strong expression of cyclooxygenase-1 and cyclooxygenase-2 proteins in the lungs of immune-competent mice (Łanocha-Arendarczyk *et al*., 2018). The current study found that two compounds of aspirin analogues included *bis*-carboxyphenyl fumarate and isopropyl *m*-bromobenzoylsalicylate were active against both cysts and trophozoites of *Acanthamoeba* spp. at lowest concentrations compared with other aspirin compounds tested in this study. The observed increase in antimicrobial activity for these compounds is associated with the structure activity relationship (the relation between the chemical structure of a molecule and its biological activity). The chemical structure for *bis*-carboxyphenyl fumarate consists of 2-formyloxy benzoic acid groups at each end connected to an ethylene group at the centre and changing ethylene group with ethyl group or simple 8 carbon chain, the compound loses its activity. Isopropyl *m*-bromobenzoylsalicylate compound is produce from propan-2-yl benzoate which linked to 3-bromobenzoic acid. This compound has a propyl group attached to benzoic acid and replacing or

eliminating the propyl group makes the compound inactive and the other aspirin based analogues studied in this analysis have different chemical structures (see Figure 4.6).

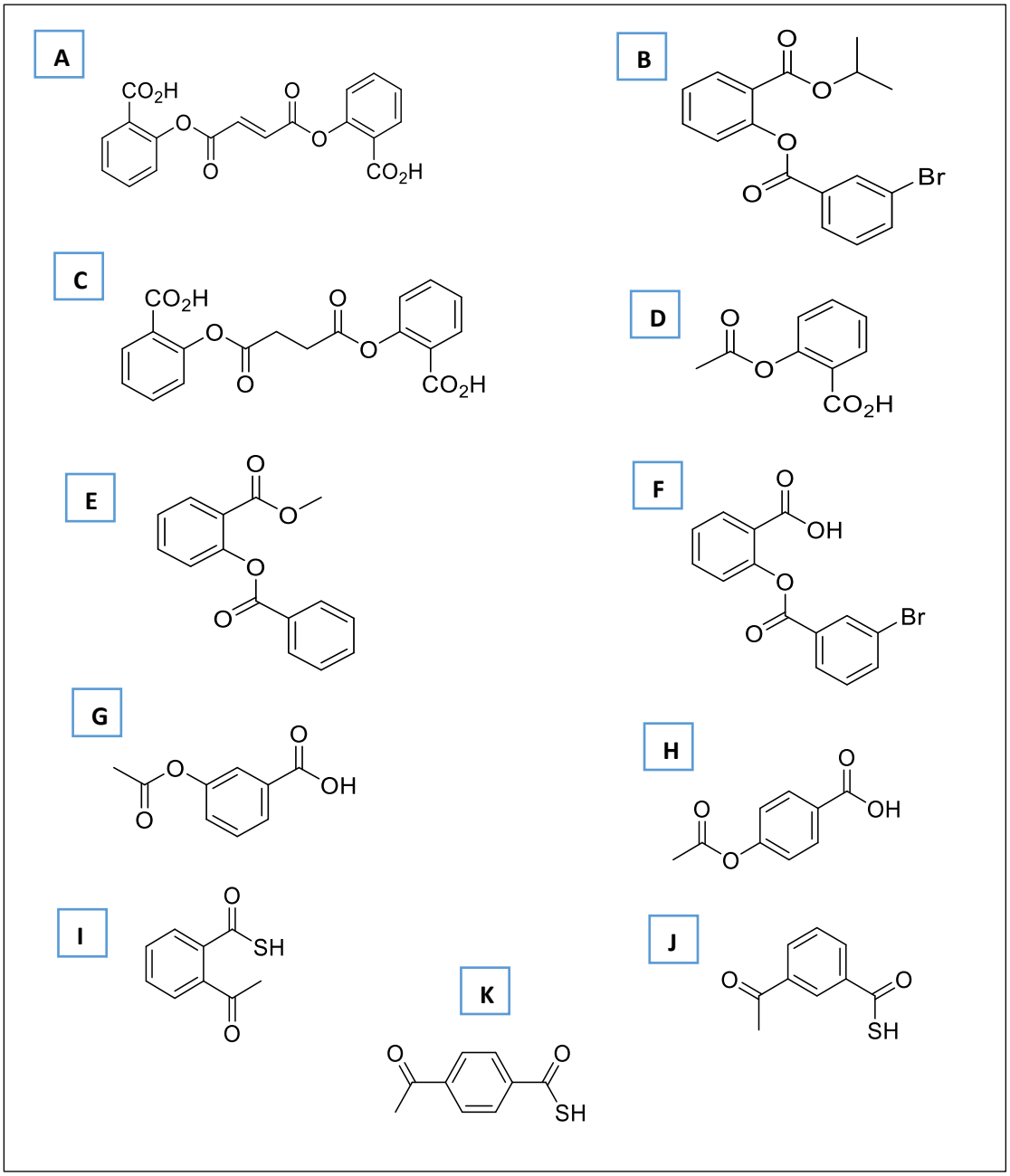

Figure 4-6: The chemical structures of (A) Bis-carboxyphenyl fumarate, (B) Isopropyl m-bromobenzoylsalicylate, (C) Bis-carboxyphenylsuccinate, (D) acetylsalicylic acid (aspirin), (E) Methyl benzoyl salicylate, (F) m-bromobenzoyl salicylic acid, (G) 3-acetoxybenzoi acid, (H) 4-acetoxybenzoic acid, (I) 2-acetylthiobenzoic acid , (J) 3-acetylthiobenzoic acid, (K) 4-acetylthiobenzoic acid.

The findings of this study are in accord with those obtained in a previous study by Claudius *et al*. (2014), which was found that the aspirin analogues inhibited the colorectal cancer cell line at a millimolar concentrations. The other aspirin analogues tested in this study found to be active against trophozoites, but a little or inactive against cysts of either species of *Acanthamoeba* at a highest concentrations from 250 µg/mL up to 500 µg/mL. The present outcomes also support Malvezi *et al*. (2014) study which concluded that aspirin analogues were inhibited the entry of *Trypanosoma cruzi* in mouse peritoneal macrophages at concentrations of 0.625 mM, 1.25 mM, and 2.5 mM and these concentrations are equivalent to 112.5 µg/mL, 225 µg/mL and 450 µg/mL respectively.

4.4.5 Efficacy of novel and existing of amidoamines on cysts and trophozoites viability

The compounds of amidoamine tested in this study, including myristamidopropyl dimethylamine (MAPD) and palmitamidopropyl dimethylamine (PAPD) were found to be active against trophozoites at between 1 and 15.6 µg/mL and against cysts at concentrations of 7.8 – 31.3 µg/mL as range for both species of *Acanthamoeba.* Among the new compounds of amidoamines, palmitoleyl-amidopropyl dimethylamine (POPD) showed greater activity against trophozoites, ranging from 3.9 to 31.3 µg/mL and against cysts at between 31.3 and 62.5 µg/mL. However, the antimicrobial activity of myristoleyl-amidopropyl dimethylamine (MOPD) against trophozoites was lower, ranging from 31.3 to 125 µg/mL and the MCC was found at the highest concentrations of 125 to 250 µg/mL. It seems possible that these results are due to the pH 6.5 of the Ac#6 medium used in the trophozoite assay, as the antimicrobial activity of MOPD was affected by the pH compared with the activity of this compound in the time kill experiment. The present compound of amidoamine, myristamidopropyl dimethylamine (MAPD) has been previously tested against cysts of *Acanthamoeba* and the MCC values were found to be between 6.25 and 25 µg/mL (Kilvington *et al*., 2002), and these results are consistent with data obtained in the current study.

4.4.6 Comparing the activity of saturated and unsaturated fatty acid derived amidopropyl amines in relation to time kill

Myristamidopropyl dimethylamine (MAPD) at a concentration of 0.0005% (w/v) formulated into a contact lens solution showed a 3-log reduction against trophozoites of *A. polyphaga* (ATCC 30461) over 24 hours. The activity increased against *A. castellanii* (ATCC 50370) and slightly more than a 3-log reduction was exhibited at the same time point. Palmitamidopropyl dimethylamine (PAPD) at the same concentration of MAPD exerted a 3-log reduction against trophozoites of both species of *Acanthamoeba*. Notably, MOPD at a concentration of 0.0005% (w/v) demonstrated complete kill against trophozoites of both species of *Acanthamoeba* at 4.5-log after 24 hours of exposure. The activity for POPD was found to be a 4.5-log reduction against trophozoites of *A. castellanii* and the activity was slightly reduced against trophozoites of *A. polyphaga* and gave 4-log reduction. There is a clear difference between the chemical structure of amidoamines and that related to the carbon chain whose length differs depending on two factors, the fatty acid source, and the functional group which gives the chemical characteristics of the compound. The novel compounds, MOPD is derived from ω-5 monounsaturated fatty acid whereas POPD is derived from ω-7 monounsaturated fatty acid. The difference between C14, C16 and C14-1, C16-1 is the branches in the carbon chain which corresponds to number (1) and this means unsaturated bonds as illustrated in Figure 3.5 below. Also, the physical properties are different, the C14 & C16 the fatty acid is solid whereas the fatty acid for C14-1 & C16-1 is liquid in room temperature, these variations are the potential reason for getting different activity against trophozoites.

Furthermore, the amidoamines possess two major functional sites for reactions which are the tertiary amine group and the amide hydrogen (Muzyczko et al., 1968). A possible explanation for the increased antimicrobial activity for the new compounds myristoleyl-amidopropyl-dimethylamine (MOPD) and palmitoleyl-amidopropyl-dimethylamine (POPD) may be related to the unsaturated bonds in their structures (Figure 4.7.C&D), which demonstrated superior activity against trophozoites when those compounds formulated into contact lenses solution compared with MAPD and PAPD as an existing compounds of amidoamine group.

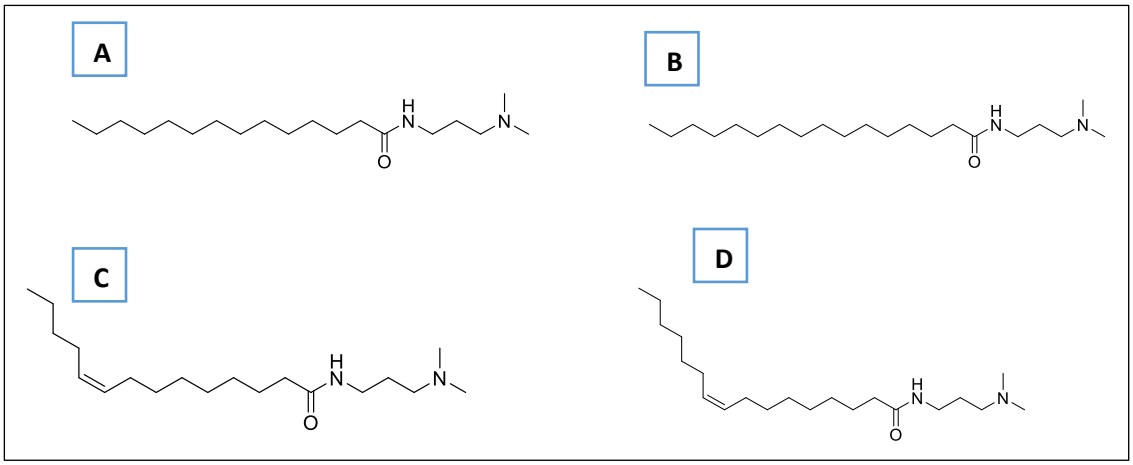

Figure 4-7: Chemical structures of (A) Myristamidopropyl dimethylamine (MAPD), (B) Palmitamidopropyl dimethylamine (PAPD), (C) Myristoleyl-amidopropyl dimethylamine (MOPD), (D) Palmitoleyl-amidopropyl dimethylamine (POPD).

It was found that the plasma membrane of *Acanthamoeba* mostly comprises of C18 and C20 lipids (Jones *et al*., 1993). As revealed in the introduction section (4.1.1). following the binding of the polar head group, the carbon chain of the surfactant is integrated into the lipid bilayer of *Acanthamoeba* and producing lateral expansion of the membrane (Denyer and Stewart, 1998). MAPD as a pure drug has been previously tested at a higher concentration of 50 mg/L against cysts of *Acanthamoeba polyphaga* and found to be active within 2 hours and showed a 4.18 log reduction (Hughes *et al.*, 2003a). The MOPD time kill findings in this study raise the possibility of using MOPD instead of MAPD as a new amidoamine compound for contact lens solutions at a concentration of 0.0005% (w/v).

4.4.7 Effect of several drugs in combination on the trophozoites viability

A single drug has not been effective against *Acanthamoeba* spp. and the treatment *in vivo* remains a major challenge. This study investigated the combined effect of 4 different drugs with chlorhexidine digluconate (CHLX) to check for the possibility of synergistic or additive effects against trophozoites of *Acanthamoeba castellanii* (ATCC 50370). The examined concentrations of the drugs were selected based on the prior MTIC trophozoite assays. According to

the present study, there no synergistic effects for the combination of two drugs were found; all four drug combinations investigated showed an additive effect against trophozoites. The CHLX had a positive impact on the pentamidine, voriconazole, natamycin and amphotericin-B. The ΣFIC for the combination of CHLX and amphotericin-B against trophozoites of *A. castellanii* (ATCC 50370) was found to be 1.14 and this value is referred to an additive effect. On the other hand, *in vitro* study by (Taravaud *et al*., 2017) on combination of CHLX with voriconazole against trophozoites of *Acanthamoeba* has reported that the ΣFIC value of 1.23 which differs to the result of ΣFIC= 2.4 obtained of the present study.

In recent an *in vitro* study conducted by Talbott *et al*. (2019) which observed that antagonistic impact from the combination of voriconazole with CHLX against cysts of *Acanthamoeba* clinical isolates. The findings of the combination of CHLX with voriconazole contradict with the combination study conducted by Kratzer *et al*. (2006) for CHLX plus voriconazole, the authors have showed that the FIC_{50} = 0.28 and FIC_{90} = 0.54 against *Trichoderma*. The FIC values reported by the authors have indicated a synergistic effect for the combination of voriconazole with CHLX against this fungi. In the present study, the combination of CHLX with the diamidine (pentamidine) against trophozoites was found to be an additive as the ΣFIC found to be 1.98 and this finding is consistent with those of (Hay et al., 1994a) who reported that the combination of pentamidine with CHLX against trophozoites of *Acanthamoeba* resulted in the additive effect. *In vitro,* it has been shown that a combination of natamycin and CHLX had a much stronger potency than monotherapy and this could be a promising treatment for *Acanthamoeba* keratitis (Kitagawa, 2003). Additionally, a combination of CHLX with carbosilane dendrimers that contains the ammonium or guanidine moieties *in vitro* had synergistic impact against trophozoites of *Acanthamoeba polyphaga* after 24 hours of incubation (Heredero-Bermejo *et al*., 2016). The current study is clearly demonstrated that the existing of CHLX enhanced the antimicrobial activity *in vitro* as the reduction against trophozoites was found to be 4-log kill compared with natamycin as a monotherapy.

4.5 Conclusion

Overall, this study tested 28 compounds against *Acanthamoeba castellanii* (ATCC 50370) and *Acanthamoeba polyphaga* (ATCC 30461) and also examined their toxicity against the human epithelial cell line. Several compounds included posaconazole, miltefosine and didecyldimethylammonium chloride were showed excellent antimicrobial activity against both cysts and trophozoites of *Acanthamoeba* spp. These compounds, therefore, need further investigation for their potential use as treatments against *Acanthamoeba* keratitis. The current study found that the novel compounds of amidoamine included myristoleyl-amidopropyl-dimethylamine (MOPD) and palmitoleyl-amidopropyl-dimethylamine (POPD) when formulated into contact lens base solution at concentration of 0.0005% (w/v) and tested against trophozoites, these compounds exhibited complete kill at 4.5 log reduction after 24 hours compared with the MAPD and PAPD as an existing compounds. This finding raises the possibility of incorporating MOPD and POPD into the contact lens solution at a concentration of 0.0005% rather than MAPD as an existing disinfectant. Among the 4 drug combinations assessed against trophozoites in this study, no synergistic effect was observed, and the findings of the current study indicated that an additive effect. The antimicrobial activity was enhanced when the CHLX combined with amphotericin–B, voriconazole, pentamidine and natamycin against trophozoites in comparison to those drugs in their own.

Chapter Five

Developing novel treatments against *Acanthamoeba* spp. and analysing the corneal penetration of chlorhexidine

Chapter 5: Developing novel treatments against *Acanthamoeba* spp. and analysing the corneal penetration of chlorhexidine

5.1 Introduction

5.1.1 Identifying new treatments against Acanthamoeba Keratitis

Many therapeutic agents have been tested against *Acanthamoeba* spp., and a number of these drugs have shown greater *in vitro* activity. However, the treatments *in vivo* against AK is still a big challenge and this is due to the fact that the findings from *in vitro* sensitivities do not always correlate with clinical outcome (Alexander *et al*., 2015). In recent years, there has been an increasing interest in used a wide range of nanoparticles-based technologies such as gold and silver as delivery vehicles to enhance the antimicrobial activity for a variety of therapeutic agents. The main reason for using the Lipodisq® carrier is because of it able to cross the lipid membrane and increase the antimicrobial activity for a different class of the drugs. Lipodisq® nanoparticles are high-density lipoproteins and range in size between 10 and 40 nm. They are considered to be a biodegradable delivery system and currently Lipodisq® nanoparticles are used as a carrier to deliver lipophilic drugs, such as fat-soluble vitamins and botanical extracts, into the outer layer of the skin (Malvern, 2015, Tonge, 2014).

The chemical structure for Lipodisq® nanoparticles are made of poly(styrene-*co*-maleic acid) (PSMA) and dipalmitoylphosphatidylcholine (DPPC) at a ratio of 1 of PSMA to 2 of DPPC (Tonge, 2014) as can be seen in Figure 5.1. There is a similarity between Lipodisq® nanoparticles and nanodiscs which are lipid protein nanoparticles and exist in high-density lipoproteins (Jonas, 1986), however, the membrane scaffold protein in nanodiscs used for the peripheral of the lipid disk, whereas a styrene/maleic acid copolymer is used as an edge for Lipodisq® nanoparticles (Zhang *et al*., 2017). The first serious discussions and analyses of nanoparticles emerged during the (1986) against different diseases, and the nanoparticles are promising drug delivery and that due to a number of factors including; their small size, cell penetration, enhancing the antimicrobial activity for drug and finally decreasing drug resistance via targeting any cellular function (Maincent *et al*., 1986).

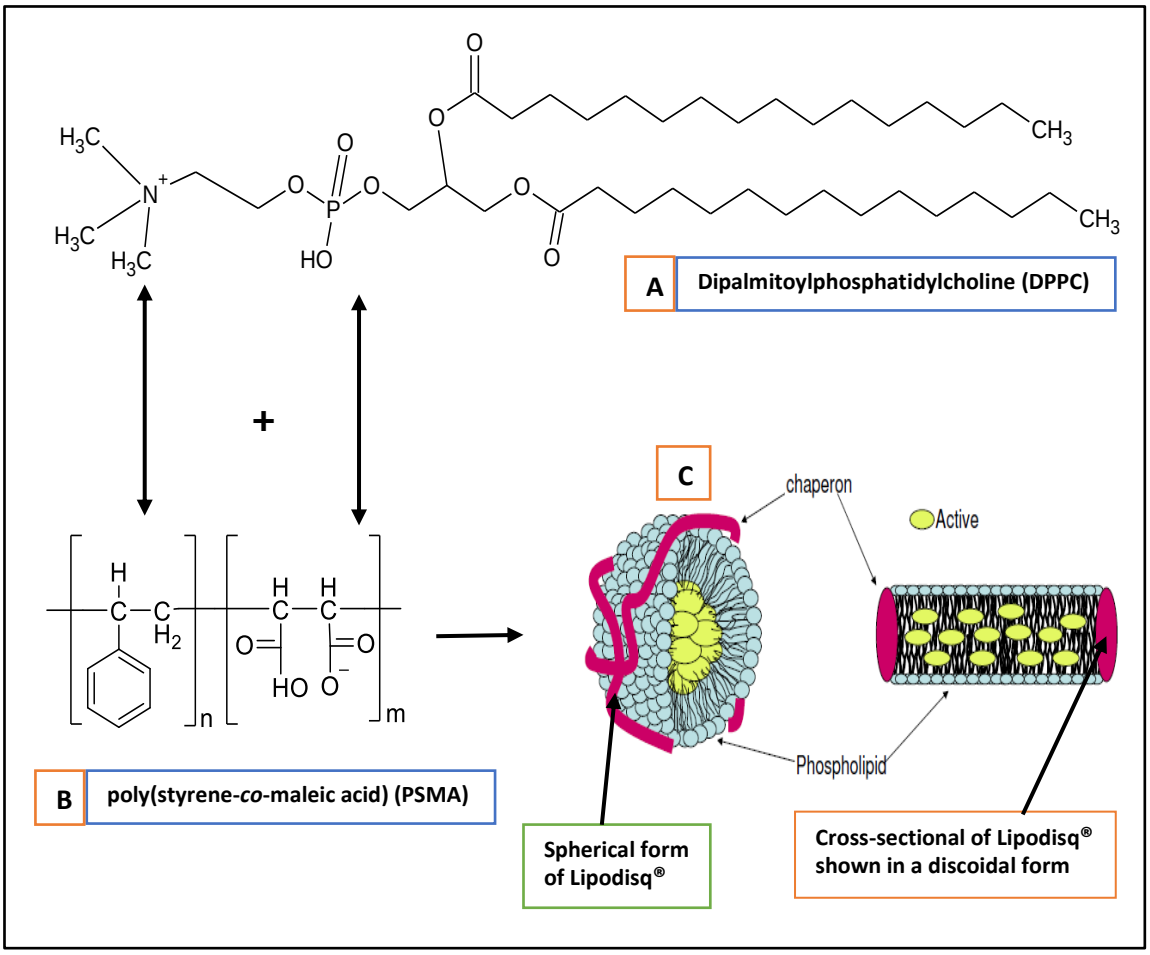

Figure 5-1: Diagram showing the synthesis of Lipodisq® nanoparticles. (A) Chemical structure of dipalmitoylphosphatidylcholine (DPPC) which represents a phospholipid and lecithin comprising of two palmitic acids linked with a phosphatidylcholine head-group. (B) Chemical structure of poly(styrene-*co*-maleic acid) (PSMA) which is made of styrene and maleic acid monomers. (C) Lipodisq® molecule exists in spherical or discoidal form[(C) adapted from (Malvern, 2015)].

The nanoparticle carriers were classified depended on two aspects firstly, how the carries are carrying the substances and secondly related to the characteristics of the matrix (Zazo *et al*., 2016). The nanoparticles were broadly divided into two main groups: organic nanoparticles (which include liposomes, polymeric nanoparticles, polymeric micelles, and solid lipid nanoparticles) and inorganic nanoparticles (which include silicon dioxide, magnetic oxide, gold and silver nanoparticles) (Zazo *et al.*, 2016, Xu *et al.*, 2006). All these carriers have played an important role for effectiveness of different kinds of drugs against many diseases. For example, for fungal infection, the incorporation of silver

nanoparticles with fluconazole had a major effect on the viability of *Candida albicans* and gave a MIC of 2.17 µg/mL compared with fluconazole alone which exhibited a MIC of >128 µg/mL after 24 hours of exposure (Longhi *et al*., 2015).

For bacterial infection, the combination of penicillin and methicillin-resistant with surface-functionalized silica nanoparticles enhanced the antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus* (Wang *et al*., 2014) Whilst for parasitic infection, the used of silver nanoparticles at a concentration of 100 µg/mL achieved 83% inhibition against *Plasmodium falciparum* (Panneerselvam *et al*., 2011). Upon incorporation of silver nanoparticles into contact lenses, superior antimicrobial activity is achieved leading to prevention of bacterial and AK infections (Willcox *et al*., 2010). Furthermore, pentamidine isethionate was incorporated into liposomes and the outcomes for this study were confirmed that a significant difference between the antimicrobial activity which was increased more than12 times than pentamidine isethionate as monotherapy for prevented *Acanthamoeba* binding to human cells (Siddiqui *et al*., 2009), also this combination was considerably more effective in inhibiting *Acanthamoeba* mediated human cell cytopathogenicity. In this chapter, a combinations of biguanide compounds with Lipodisq® carriers have been made for the first time to establish their antimicrobial activity against trophozoites and cysts of *Acanthamoeba* and to compare the activity with the use of biguanides alone. As reviewed in chapter 1 of this thesis subheading (1.14) that the mechanism of action for biguanides producing damage in the membranes of *Acanthamoeba.* PHMB and chlorhexidine are positively charged and interfering with mucopolysaccharide plug of the ostiole and causing penetration of the *Acanthamoeba* (Khunkitti *et al*., 1997). Subsequent, these drugs bind to the phospholipid bilayer of the cell membrane, which is negatively charged, causing structural and permeability changes, ion leakage, cytoplasmic cell damage and cell death (Lim *et al*., 2008a).

5.1.2 Penetration of chlorhexidine into the corneal tissue

Corneal drug penetration evaluation is important for the development of effective ophthalmic medications. Several methods utilised to enhance the ocular bioavailability (including pre-corneal drug retention, the use penetration enhancers, liposomes, micro-and nanospheres) have been previously investigated (Agarwal and Rupenthal, 2016, Krishnaswami *et al*., 2018). It has been revealed in the literature that, three distinct concentrations of CHLX at 0.02% (v/v). 0.1% (v/v) and 0.2% (v/v) were used to study the penetration profile of corneal and anterior chamber of rabbit, and the findings of this investigation revealed that the mean corneal of CHLX concentration was 0.28 µg/mg, 0.105 µg/mg and 0.575 µg/mg respectively, so the CHLX can penetrate across the intact cornea (Banich *et al*., 2003). The penetration of CHLX combined with Lipodisq® nanoparticles was investigated as it is used for different applications such as skin penetration, it was reported that the Lipodisq® carrier improved the pentation of the skin compared with CHLX alone (Karpanen *et al*., 2008). To date, a recent publication has assessed the penetration into corneal tissue of chlorhexidine (CHLX) in eye drops at a concentration of 0.2% (v/v) and 2% (v/v) of CHLX combined with β-cyclodextrin as a carrier. The findings showed that the β-cyclodextrin had a major impact on improving the penetration of CHLX: the volume of CHLX plus β-cyclodextrin increased 5-fold compared with CHLX alone after 2 hours of penetration (Hewitt *et al*., 2020).

5.1.3 Aims and objectives of this chapter

AK is a concerning disease which still fails to respond to many antimicrobial agents. The description of the development of a new formulation of biguanides incorporated with Lipodisq® nanoparticle carriers is one of the aims of this chapter. These compounds were tested against cysts and trophozoites of *Acanthamoeba* spp. The antimicrobial kinetics for the novel combinations are then evaluated and the toxicity of the novel compounds against human epithelial cell line are determined. Finally, the penetration profile into cornea tissue of Lipodisq® carries combined with chlorhexidine is examined. The objectives of this chapter are as follows:

I. The testing of biguanide compounds combined with Lipodisq® carries against trophozoites and cysts of *Acanthamoeba* spp.
II. Performing a time kill study for the combination of biguanides with Lipodisq® and the existing biguanides against cysts of *Acanthamoeba* spp. and comparing the activity of the novel combination with existing compounds of biguanide.
III. Assessing the toxicity of the biguanide compounds plus Lipodisq® and the current compounds against the human epithelial cell line.
IV. Investigating the capacity of the Lipodisq® nanoparticle carriers in enabling trans-corneal penetration of the hydrophilic drug chlorhexidine into the lipophilic cornea tissue.

5.2 Materials and Methods

5.2.1 Preparation of organisms

The *Acanthamoeba castellanii* (ATCC 50370) and *Acanthamoeba polyphaga* (ATCC 30461) test organisms were grown and prepared according to the methods detailed in chapter 2, sections 2.4–2.8.

5.2.2 Novel and existing drugs sensitivity testing assays

The current biguanide drugs used to treat AK were tested in their own in this study and then incorporated with Lipodisq® nanoparticle carries as novel combinations and assessed their activity against trophozoites and cysts of *Acanthamoeba* spp.

Table 5-1: List of novel formulation of Lipodisq® with biguanides and the existing of biguanides, class/use and solubility which they were tested for their antimicrobial activity and toxicity.

Drug	Class/Use	Solubility
Octenidine hydrochloride (existing drug)	Quaternized pyridine	H_2O
Polyhexamethylene biguanide (PHMB) (existing drug)	Polymeric Biguanide	H_2O
Chlorhexidine digluconate (CHLX) (existing drug)	Biguanide	H_2O
CHLX + Lipodisq® (novel formulation)	Biguanide	H_2O
Octenidine + Lipodisq® (novel formulation)	Pyridine / Cationic antiseptic	H_2O
PHMB + Lipodisq® (novel formulation)	Polymeric Biguanide	H_2O
PHMB + Lipodisq® non-particle (LQNP) (novel formulation): The LQNP means the solution formulation contains a polystyrene-maleic acid (PSMA) in the absence of dipalmitoylphosphatidylcholine (DPPC)	Polymeric Biguanide	H_2O
PHMB + Surfactant (novel formulation): The surfactant means the solution formulation comprises of polysorbate 20 (Tween 20) instead of PSMA and performs a similar role to the PSMA by interacting with (DPPC)	Polymeric Biguanide	H_2O
CHLX + Surfactant (novel formulation)	Biguanide	H_2O
CHLX + Lipodisq® non-particle (novel formulation)	Biguanide	H_2O

The novel biguanides as stock solutions, included PHMB incorporated with Lipodisq® at a concentration of 0.6% or CHLX and octenidine combined with Lipodisq® at a concentration of 0.1% were obtained from our collaborators in Malvern, Worcestershire, U.K. The other formulations involved Lipodisq® non-particle and surfactant were prepared in our laboratory by combining the existing compound solutions of PHMB or CHLX with Lipodisq® nanoparticle carrier solution or surfactant solution at ratios of 1 : 199 to give a final concentrations of 1 mg/mL. All formulations were mixed by vortexing and were subsequently assayed to determine their antimicrobial activity against trophozoites and cysts of both species of *Acanthamoeba* as outlined in chapter 2, sections 2.11–2.13, in order to determine the Minimum Trophozoite Inhibitory Concentration (MTIC), the Minimum Trophozoite Amoebicidal Concentration (MTAC) and the Minimum Cysticidal Concentration (MCC) for each of the compounds. Formulations were considered as a suitable candidates if they had the same or greater antimicrobial activity as the compounds that are now used in the treatment of AK.

5.2.3 *In vitro* toxicological testing against human epithelial cell line

After determining the antimicrobial activity for all compounds against *Acanthamoeba* spp. a different *in vitro* assay was performed to determine the toxicological profile for each of the formulations against a human epithelial cell line. The human epithelial cell line was maintained and prepared as outline in chapter 2, section 2.16–2.18.

5.2.4 Time kill assays for novel and existing biguanides

The time kill experiments where performed for novel and existing biguanide compounds which they showed excellent antimicrobial activity against Neff's cysts and trophozoites of *Acanthamoeba* spp. The reduction in viable cysts or trophozoites were plotted as change in log viability for each time point compared to zero-time viability, as described in Chapter 2, Section 2.13.

5.2.5 *Ex vivo* corneal penetration

For these experiments whole eyes were removed from pigs shortly after slaughter and transported to the laboratory for testing. The eyes were obtained from Gill's slaughterhouse in Wolverhampton and were taken from animals that were being processed for the food industry making use of material that would otherwise be discarded. The entire eyes were incubated anterior side down in the test solution into the wells of a 6-well plate, the well containing 5 mL of phosphate buffer saline (PBS) as control or chlorhexidine (CHLX) and chlorhexidine combined with Lipodisq® carrier at a concentration of 0.02% (v/v) with care taken to make sure the level of solution did not exceed the limbus which is the interface between the cornea and the sclera (the white part of the eye). Subsequently, the eyes were processed in duplicate and incubated for 10 min and 1 hour in either CHLX or CHLX combined with Lipodisq® nanoparticle carrier. After incubation the corneas were removed from the rest of the eye using a surgical blade and curved scissors (SLS, Nottingham, U.K.) and then stored at -80°C until HPLC testing was performed.

5.2.6 High Performance Liquid Chromatography (HPLC) analysis

The penetration into the corneal tissue of CHLX and CHLX incorporated with Lipodisq® carriers was determined by HPLC analysis. When the incubation time was complete for CHLX and CHLX plus Lipodisq®, the corneal tissue was prepared for HPLC analysis by incubating the tissue samples in HPLC mobile phase solution and then heated to 60°C for 1 hour as previously described by (Karpanen *et al*., 2008). The separation method was performed by using C18 column in the mobile phase comprised methanol : water (HPLC grade) 75:25 (v/v) with 0.005 M of sodium heptane sulfonate and 0.1% (v/v) of diethylamine. The pH was adjusted to 4 by using glacial acetic acid. The detection wavelength was set at 254 nm, the injection volume at 10 µL and the flow rate at 1.5 mL/min. Under these conditions, CHLX and CHLX plus Lipodisq® were found to have a retention time of 2.513 and 2.502 min respectively.

5.2.7 Ethics statement

The corneal tissue from pig eyes was approved by the Life Sciences Ethics Committee, Faculty of Science and Engineering, University of Wolverhampton and the approval number is: LSEC/201920/WH/111.

5.3 Results

The results in this chapter are presenting the novel biguanides formulations testing along with the current treatments and also the HPLC findings for the penetration of chlorhexidine as a monotherapy and in combination with Lipodisq® carrier into the corneal tissue as an *ex vivo* models.

5.3.1 Biguanides combined with Lipodisq® nanoparticle carriers

PHMB alone was demonstrated a lower antimicrobial activity ranged 7.8 – 15.6 µg/mL against cysts and between 0.48 – 3.9 µg/mL against trophozoites for both species. Interestingly, when the PHMB incorporated with Lipodisq® it shows higher activity ranged from 0.24 – 0.48 µg/mL against cysts for either species, the amoebicidal activity was 0.97 µg/mL for both species. PHMB combined with Lipodisq® was more tolerated to the human epithelial cell line at MCT 125 µg/mL than PHMB as its own which was demonstrated MCT 31.3 µg/mL. The other two combinations were showed a higher activity against trophozoites ranged 0.48 – 0.97 µg/mL for both species and between 1.95 – 3.9 µg/mL against cysts for either species, also these two compounds were toxic to the human epithelial cell line at MCT 1.95 µg/mL (Table 5.2). CHLX as monotherapy showed minor cysticidal activity with MCC 31.3 µg/mL and the amoebicidal activity ranged from 7.8 – 15.6 µg/mL, also CHLX on its own was slightly toxic to human cell line at MCC 3.9 µg/mL. When the CHLX was combined with Lipodisq® carrier the antimicrobial has enhanced against cysts of both species of *Acanthamoeba* with MCC ranged from 1.95 – 3.9 µg/mL as well as the antimicrobial activity has increased against trophozoites between 0.48 – 0.97 µg/mL for both species. This compound was not toxic to human epithelial cell line with MCT 31.3 µg/mL.

The other two combinations of CHLX were showed a significant activity against trophozoites for both species ranged from 0.97 – 3.9 µg/mL, but the activity lower down when they were tested against cysts for either species ranged from 15.6 – 31.3 µg/mL. These two formulations were toxic to human epithelial cell line at

MCT between 1 – 1.95 µg/mL. In this study the Lipodisq® in its own was tested against both species of *Acanthamoeba* and no activity was observed. Also, when the Lipodisq® was assessed on the human epithelial cell line it gave MIC 62.5 µg/mL and MCT 125 µg/mL, so it was not toxic to the human epithelial cell line. The octenidine hydrochloride (OCT) was tested alone and it exhibited activity against cysts between 3.9 – 7.8 µg/mL as a range against either species. A great activity was observed against trophozoites for OCT ranged from 0.5 – 1.95 µg/mL for both species. When the OCT combined with Lipodisq® carriers, the antimicrobial activity against cysts was increased as the activity observed between 1.95 µg/mL and elevated to 3.9 µg/mL. The activity for OCT against trophozoites was ranging from 0.48 – 1.95 µg/mL against each of the tested species. The toxicity test showed that the OCT alone was toxic to the human epithelial cell line at MCT of 1.95 µg/mL. However, the combination of OCT with the Lipodisq® carriers demonstrated a little toxicity on the human cell line as the MCT value was found to be at 15.6 µg/mL (Table 5.2).

Table 5-2: Efficacy of antimicrobials in the presence of lipodisq nanoparticles against trophozoites and cysts for *A. polyphaga* & *A. castellanii* and for their toxicity against a human epithelial cell line (Hep2).

	In vitro drug sensitivities (µg/mL)							
	A. castellanii (ATCC 50370)			*A. polyphaga* (ATCC 30461)			Hep2	
Drug	MTIC*	MTAC**	MCC***	MTIC*	MTAC**	MCC***	MIC+	MCT++
Octenidine hydrochloride alone	1	1.95	7.8	0.5	1	3.9	3.9	1.95
Octenidine combined with Lipodisq®	0.97	1.95	3.9	0.48	0.97	1.95	7.8	15.6
Polyhexamethylene biguanide (PHMB) alone	1	3.9	15.6	1	7.8	7.8	62.3	31.3
PHMB combined with Lipodisq® non-particle	0.48	0.97	3.9	0.48	0.97	1.95	3.9	7.8
PHMB combined with surfactant	0.48	0.97	1.95	0.48	0.97	1.95	3.9	15.6
PHMB combined with Lipodisq®	0.48	0.97	0.48	0.48	0.97	0.24	62.3	125
Chlorhexidine digluconate (CHLX)	1.95	7.8	31.3	3.9	15.6	31.3	7.8	15.6
CHLX combined with Lipodisq®	0.48	0.97	3.9	0.48	0.97	1.95	7.8	31.3
CHLX combined Lipodisq® non-particle	0.97	3.9	7.8	0.97	3.9	15.6	3.9	7.8
CHLX combined with Surfactant	0.97	1.95	15.6	0.48	3.9	7.8	7.8	15.6
Lipodisq alone as a control	>500	>500	>500	>500	>500	>500	62.5	125

MTIC* Minimum trophozoite inhibitory concentration, MTAC** Minimum trophozoite amoebicidal concentration, MCC*** Minimum cysticidal concentration, MIC+ Minimum inhibitory concentration, MCT++ Minimum Cytotoxic Concentration. The testing was carried out with the other forms of the Lipodisq® included non-particle and surfactant in order to verify the activity of Lipodisq® in the presence and absence of dipalmitoylphosphatidylcholine and poly(styrene-*co*-maleic acid).

5.3.2 Time kill studies for biguanides

After determining the MCC for the new biguanide formulations using a microtitre plate, the next step was to examine the kinetics of those compounds against Neff's cysts of *Acanthamoeba* utilizing time-kill experiments, as shown in Figures 5.2, 5.3 & 5.4 below.

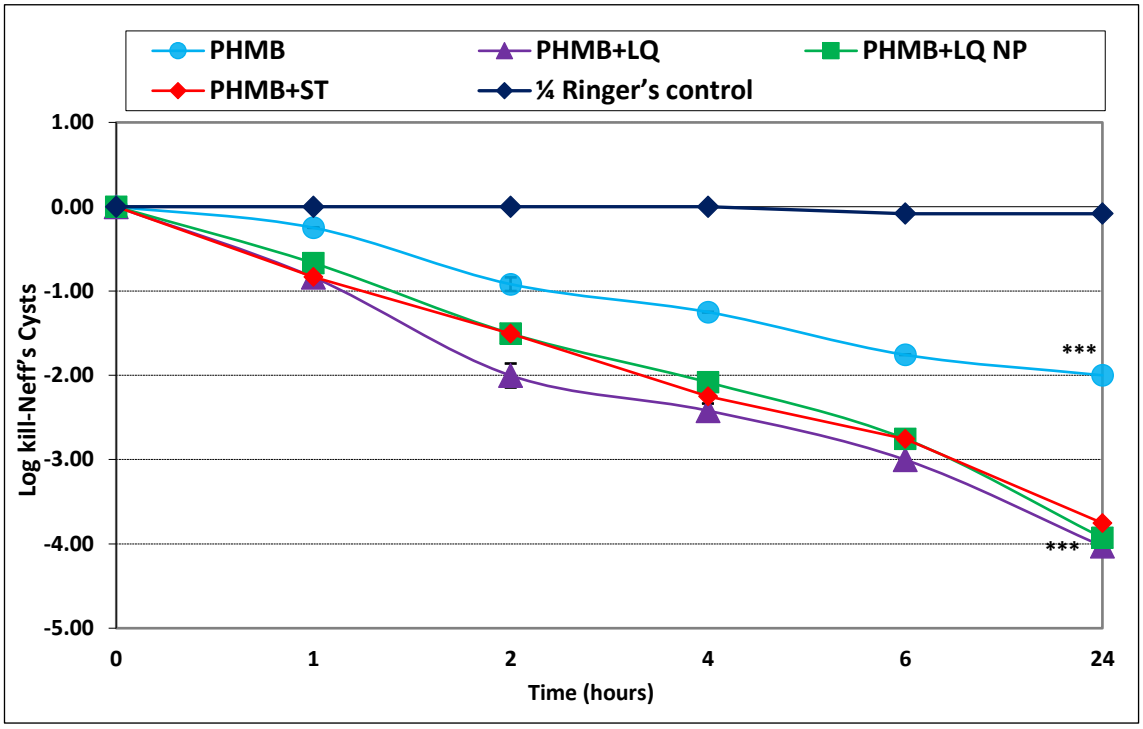

Figure 5-2: Effect of PHMB, PHMB plus Lipodisq® (PHMB+LQ), PHMB Lipodisq® non-particle (PHMB+LQ NP) and PHMB plus surfactant (PHMB+ST) at concentration of 31.3 µg/mL against Neff's cyst of *Acanthamoeba castellanii* (ATCC 50370). One-way analysis of variance (ANOVA) was performed. Asterisks represent values statistically significant (***P<0.001) between the tested combinations of PHMB and the ¼ strength Ringer's control.

PHMB combined with Lipodisq®, PHMB Lipodisq® non-particle and PHMB combined with surfactant were showed a comparable log reduction at ≈4-log kill against cysts of *A. castellanii* (ATCC 50370) after the 24 hours of the experiment. PHMB as monotherapy exhibited moderate activity against cysts of the same strain, giving only 2-log kill over 24 hours. One Way ANOVA analysis showed that PHMB plus Lipodisq®, PHMB Lipodisq® non-particle and PHMB plus surfactant were statistically significant ($P < 0.001$) over 24 hours compared to the PHMB alone and ¼ strength Ringer's as control (Figure 5.2). The means after 24

hours for all evaluated compounds in relation to the ¼ strength Ringer's control were subjected to two sample t-test analysis and the p-value was observed at p=0.0105 and this value is less than 0.05.

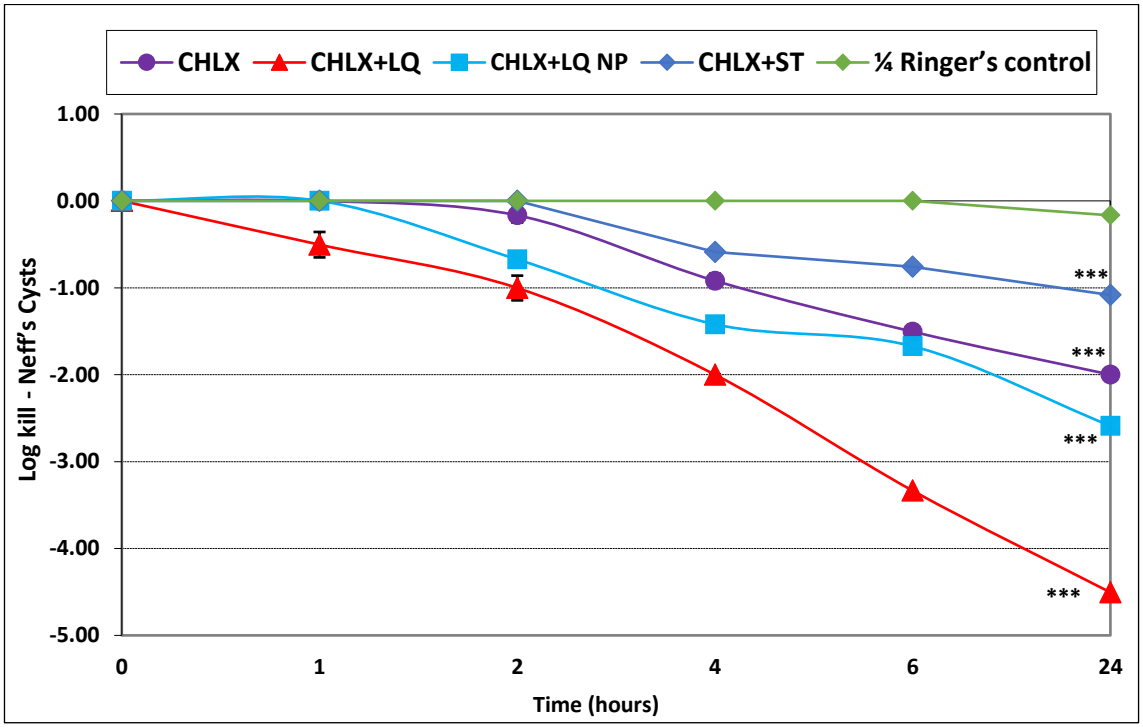

Figure 5-3: Effect of CHLX, CHLX combined with Lipodisq® (CHLX+LQ), CHLX plus Lipodisq® non-particle (CHLX+LQ NP) and CHLX plus surfactant (CHLX+ST) at a concentration of 15.6 µg/mL against Neff's cyst of *Acanthamoeba castellanii* (ATCC 50370). One-way analysis of variance (ANOVA) was conducted. Asterisks represent values statistically significant (***P<0.001) between the tested combinations of CHLX and the ¼ strength Ringer's control.

Interestingly, over the 24 hours of the experiment, CHLX combined with Lipodisq® was found to be highly active against Neff's cysts of *A. castellanii* providing a 4.5-log reduction. During 24 hours of exposure, CHLX encapsulated with Lipodisq® was statistically significant (P < 0.001) in terms of the antimicrobial activity in comparison with ¼ strength Ringer's control and other combinations of CHLX (Figure 5.3). Further statistical analysis, two sample t-test showed that the p-value (p=0.0197) for all tested combinations compared to the ¼ strength Ringer's control.

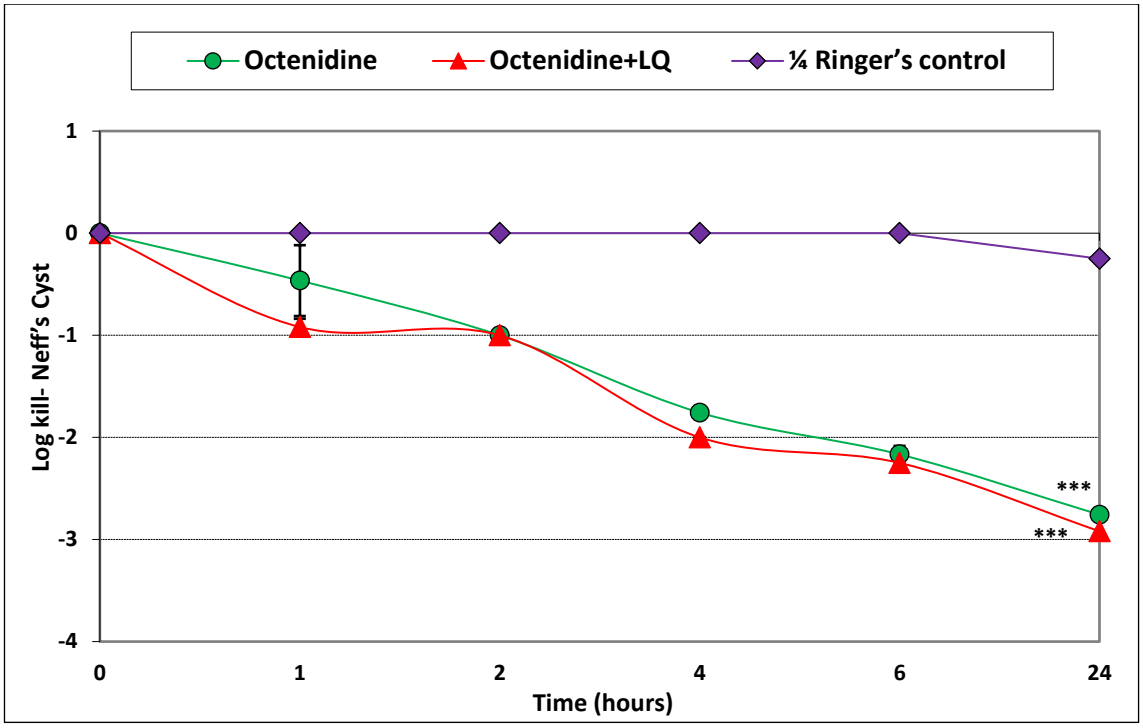

Figure 5-4: Effect of octenidine hydrochloride at a concentration of 7.8 µg/mL and octenidine hydrochloride combined with Lipodisq® carrier at a concentration of 3.9 µg/mL against Neff's cysts of *Acanthamoeba castellanii* (ATCC 50370). One-way analysis of variance (ANOVA) was performed. Asterisks represent values statistically significant (***P<0.001) between the tested concentrations of octenidine or octenidine plus Lipodisq® and the ¼ strength Ringer's control.

After the 24 hours of the time kill experiment, octenidine incorporated with Lipodisq® carriers at a concentration of 3.9 µg/mL achieved a 3-log kill against Neff's cysts of *A. castellanii*, whereas octenidine in its own and at a concentration of 7.8 µg/mL, gave ≈3 log reduction at the same time point. There was no significant difference between octenidine combined with Lipodisq® and octenidine hydrochloride used as a monotherapy at the 24-hour time point as the two sample t-test showed that the p-value (p=0.0717) and this is more than 0.05, but a significant was observed for octenidine alone and octenidine plus Lipodisq® relative to the ¼ strength Ringer's control with a p-value of (p=0.0210) (Figure 5.4).

5.3.3 Corneal penetration investigations

The Lipodisq® nanoparticle carrier is currently used to improve the delivery of hydrophilic drugs into the skin as it has the ability to traverse the lipid membranes of intact cells. Our anticipation was that this carrier would be able to deliver the hydrophilic compound CHLX into the corneal tissue that would be highly important for its use in the treatment of AK. Our *in vitro* findings of drugs screening showed that the antimicrobial activity for CHLX was enhanced in the presence of Lipodisq® nanoparticle carrier against trophozoites and cysts of *Acanthamoeba* spp. The results from the drug testing of this study indicated that the antimicrobial activity for biguanides has increased in the presence of the Lipodisq® carrier against cysts and trophozoites and these results are likely to be related to the ability of Lipodisq® nanoparticle carrier to cross the lipid membrane of *Acanthamoeba*.

Initially, a standard curve of CHLX as monotherapy was created to enable the quantification of the CHLX extracted from the corneal tissue using a range of concentrations from 200 µg/mL to 2 µg/mL. After that a calibration curve was generated by plotting the peak area of CHLX analysis against CHLX concentration for each of the standards from Table 5.3.

Table 5-3: The corresponding peak area obtained for each of the CHLX standards

Standard	Concentration (µg/mL)	Peak Area
1	200	7454578
2	100	3780456
3	50	1829043
4	10	386586
5	5	159336
6	2	63120

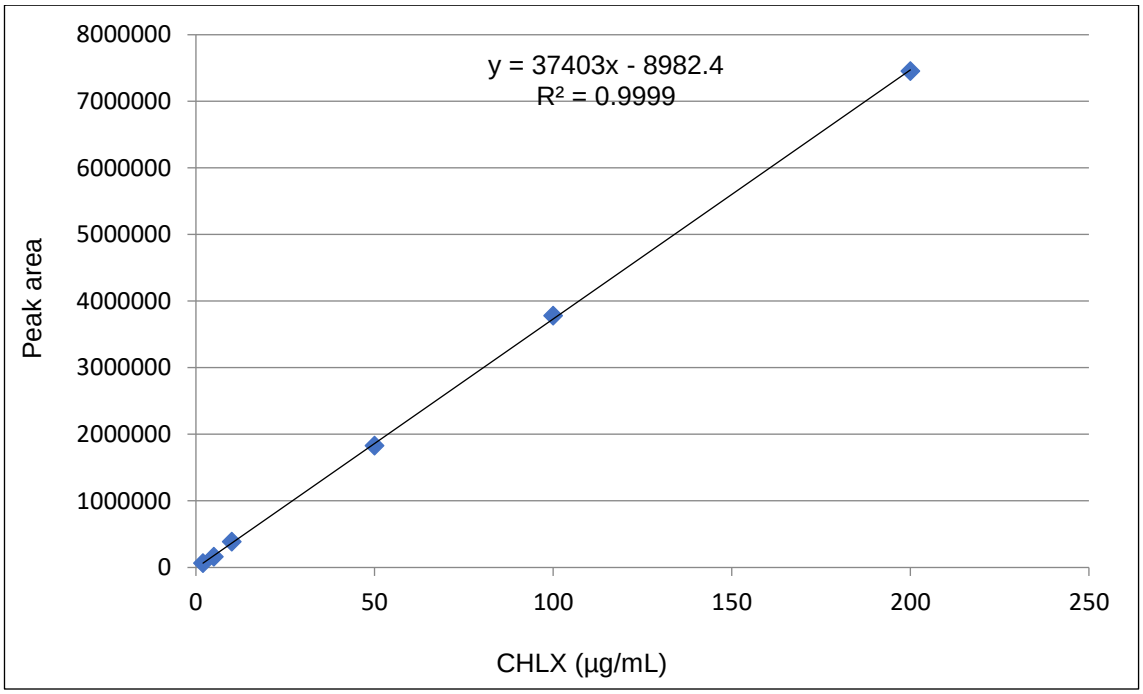

Figure 5-5: Calibration curve showing the peak area against CHLX concentrations

It is clear from the HPLC analysis that the CHLX alone showed a major peak at retention time of 2.513 min as shown in Figure 5.6. The CHLX incorporated with Lipodisq® nanoparticle peak was observed in comparable retention time with CHLX in its own at 2.502 min, see Figure 5.7. The other detected peaks in the chromatogram may related to noise and they are compounds that could be impurities or from the corneal tissue itself.

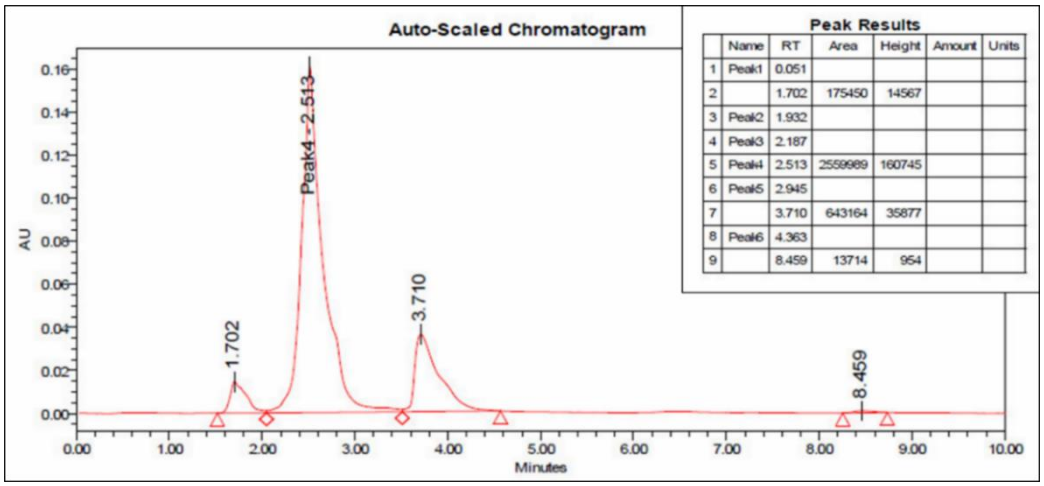

Figure 5-6: Chromatogram shows the analysis of the CHLX alone concentration corneal tissue which had been incubated for 1 hour in 200 µg/mL of CHLX.

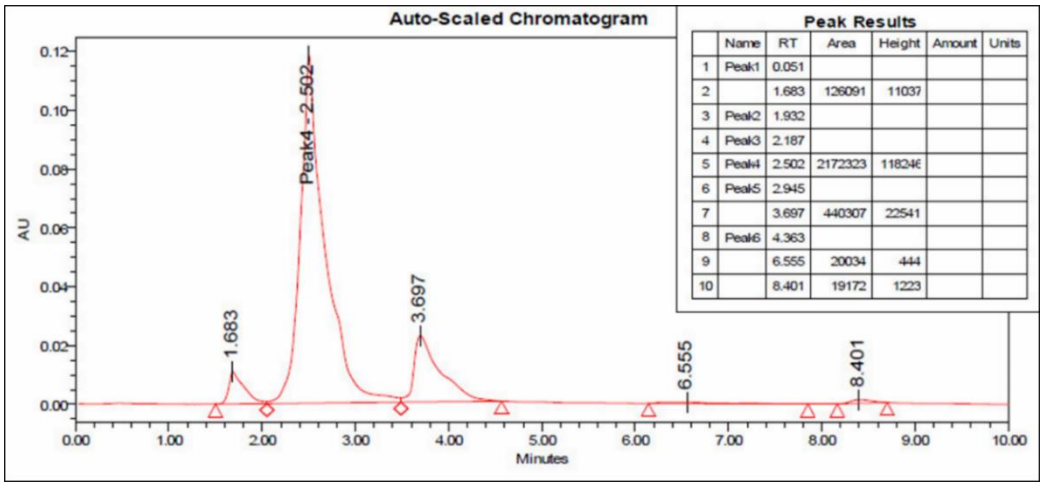

Figure 5-7: Chromatogram shows the analysis of the CHLX concentration corneal tissue which had been incubated for 1 hour in 200 µg/mL of CHLX combined with Lipodisq® nanoparticle carrier.

The CHLX in the presence of the Lipodisq® nanoparticle (the initial of exposure concentration to the cornea is 0.02% (v/v) demonstrated a lower concentration at 10.8 µg/mL, whereas the concentration was increased up to 16.3 µg/mL in the absence of the carrier at the same exposure concentration that used for CHLX + carrier as shown in Table 5.4 below. So, the results of this study suggested that the Lipodisq® nanoparticle carrier was not penetrated deeply inside the corneal tissue compared with CHLX alone and the possible reason for that is the limitation of the detected method.

Table 5-4: The quantification of the CHLX concentration from the corneal tissue exposed to CHLX in the presence and absence of the Lipodisq® nanoparticle carrier. The standard curve from Figure 5.5 was used to determine the concentration of CHLX.

Sample	Time	Peak Area	Concentration (µg/mL)
CHLX as monotherapy	10 min	308966	8.5
CHLX combined with Lipodisq®	10 min	262195	7.3
CHLX as monotherapy	1 hour	643164	16.3
CHLX combined with Lipodisq®	1 hour	440307	10.8

5.4 Discussion

5.4.1 Increases in the activity of biguanides in the presence of Lipodisq® nanoparticles

Most of the studies in the scientific literature have reported that the first therapy line used for the treatment of AK is biguanides, either PHMB or CHLX as monotherapies at concentration of 0.02 % (v/v) or in combination with diamidine compounds, such as propamidine isethionate and hexamidine at a concentration of 0.1% (v/v) (Elder *et al*., 1995, Bouheraoua *et al*., 2014, Papa *et al.*, 2020). Chlorhexidine is bisbiguanides which has previously been tested and it showed strong activity against a number of bacterial species, the minimal bactericidal concentration (MIC) for *Streptococcus mutans* found to be 1.60 mM and a much lower concentration of MIC was observed at 0.40 mM for other strains included *S. sanguis*, *Actinomyces viscosus* and *A. naeslundii* (Tanzer *et al*., 1977). PHMB is a biguanide polymer and is comprised of repeating simple biguanide units linked with hexamethylene hydrocarbon chains (Chindera *et al*., 2016).

PHMB is a broad-spectrum biocide that is used to eradicate a number of microorganisms, such as parasites, bacteria, fungi and some viruses (Müller and Kramer, 2008). The chemical structure of biguanide compounds comprises two or more biguanide groups and a lipophilic 6-carbon chain (Figure 5.8). One of the most interesting observations is that PHMB, CHLX and octenidine, when incorporated with Lipodisq® nanoparticle carriers, demonstrated excellent antimicrobial activity against cysts and trophozoites of both species of *Acanthamoeba*. The antimicrobial activity was enhanced in the presence of Lipodisq® carriers. This may be due to several reasons including the following: the carrier is able to target the cell function and reduce drug resistance; it is easier to penetrate inside the cell and its small size enables the carrier to cross the lipid membranes of intact cells.

To our knowledge, no study has tested a combination of PHMB, CHLX and octenidine with Lipodisq® nanoparticle carriers against trophozoites and cysts of *Acanthamoeba,* so this is first study to show the antimicrobial activity of these combinations against trophozoites and cysts. Previously, PHMB and CHLX have been tested *in vitro* against cyst of *Acanthamoeba castellanii* and both drugs showed antimicrobial activity at a higher concentration of 25 µg/mL (Khunkitti *et*

al., 1996). Conversely, the results from the combination of PHMB and CHLX with Lipodisq® carrier in the current study suggested that the activity of PHMB is enhanced in the presence of this carrier up to around 7-fold and also 4-fold increase was observed for CHLX against cysts.

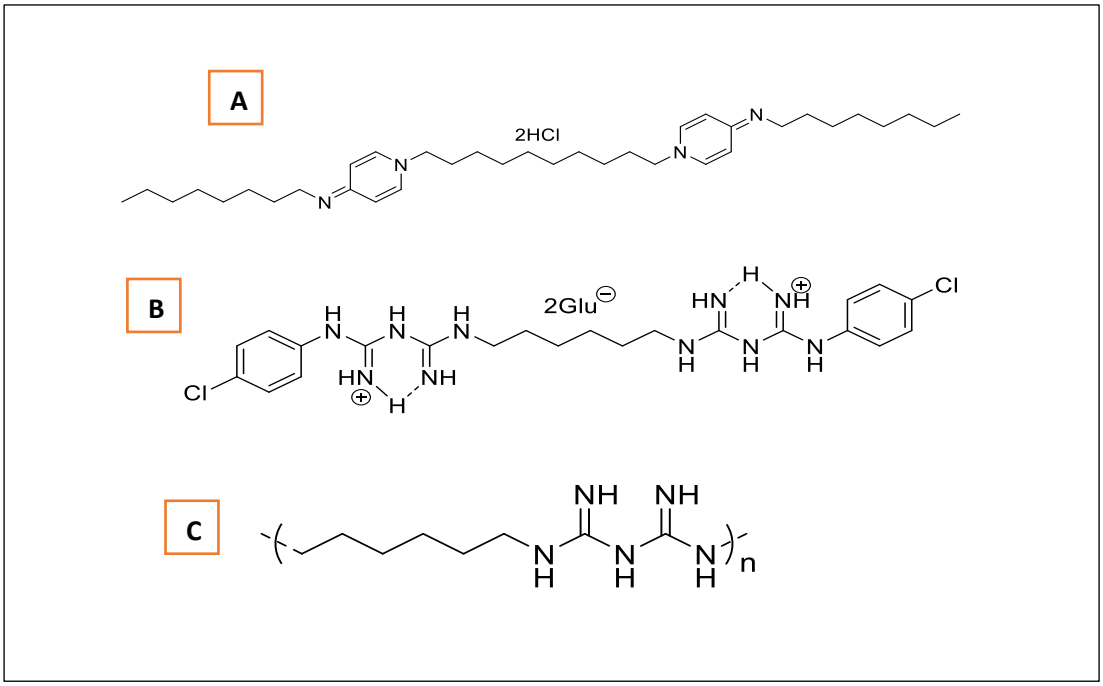

Figure 5-8: Chemical structures of biguanide compounds: (A) Octenidine hydrochloride, (B) chlorhexidine, (C) polyhexamethylene biguanide

The Lipodisq® nanoparticle solutions were formulated by our collaborators in Malvern, Worcestershire, U.K. Essentially, the formulation was made through the synthesis of lipodisq nanoparticles from poly(styrene-*co*-maleic acid) and dipalmitoylphosphatidylcholine (DPPC) at ratio of 1:2 (Tonge, 2014). The lipodisq particles size was monitored by using dynamic light scattering and then the Lipodisq® solution was combined with CHLX and octenidine hydrochloride at a concentration of 0.1% (v/v) and with PHMB at a concentration of 0.6% (v/v). The other new formulations of surfactant and Lipodisq® non-particle were combined with CHLX or PHMB at a ratio of 1:199 (Figure 5.9).

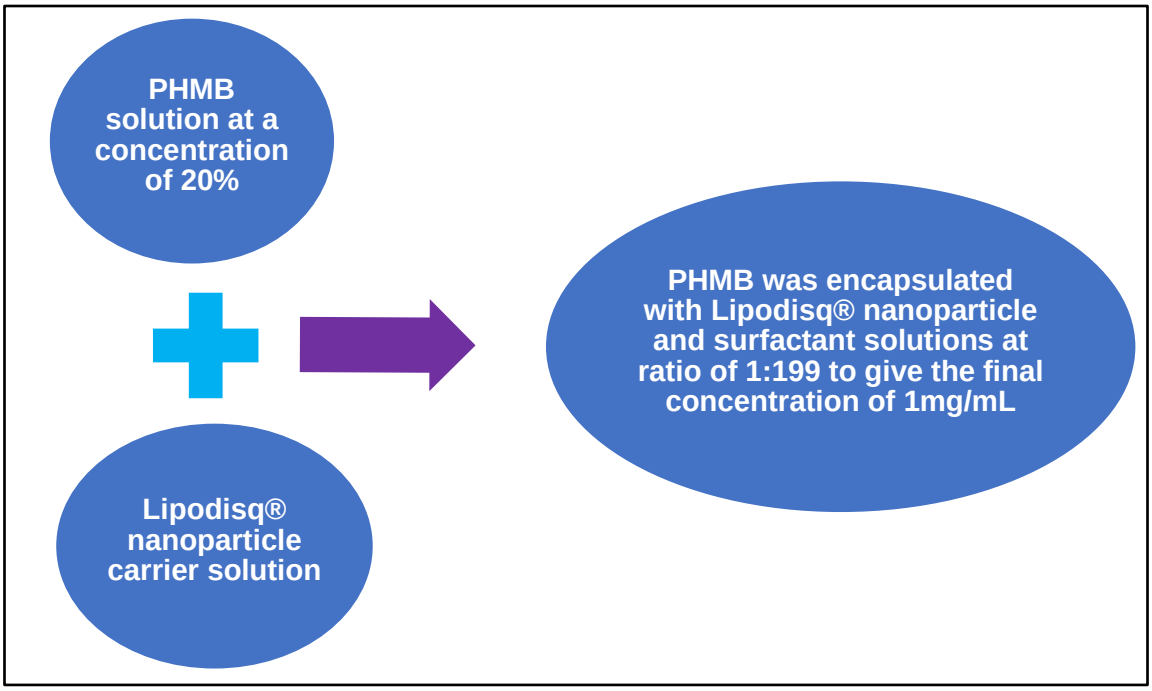

Figure 5-9: Formulation of a Lipodisq® nanoparticle solution with biguanides, such as polyhexamethylene biguanide (PHMB).

A different carrier of gold nanoparticles was incorporated with chlorhexidine and tested against trophozoites of *Acanthamoeba* and the findings showed that at a lower concentration of 5 µM caused 60% of inhibition compared with chlorhexidine alone which exhibited only 40% of inhibition against trophozoites (Bouheraoua *et al.*, 2014). The antimicrobial activity observed in the existence of Lipodisq® nanoparticles against trophozoites and cysts in this study is very interesting. The way forward may be to apply a combination of biguanide compounds with Lipodisq® carriers in a clinical trial setting. However, it must be remembered that there is no association between *in vitro* drug sensitivities and the *in vivo* response of most of the drugs used for the treatment of AK (Pérez-Santonja *et al*., 2003).

5.4.2 Evaluating the activity of new formulation and existing of biguanides in relation to time kill

Polyhexamethylene biguanide (PHMB), chlorhexidine digluconate (CHLX) and octenidine hydrochloride (OCT) were evaluated on their own and then encapsulated with nanoparticles Lipodisq® carriers to establish their antimicrobial activity over time against cysts of *Acanthamoeba*. It is clear from the time kill results for biguanides that a large improvement was observed in antimicrobial

activity against Neff's cyst of *Acanthamoeba castellanii* (ATCC 50370). There was a large increase in the activity of biguanides against both trophozoites and cysts of *A. castellanii* in the presence of Lipodisq® nanoparticle carriers in comparison with the absence of such carriers. As presented earlier in the result section 5.3.4 of this chapter, PHMB and CHLX plus Lipodisq® at a concentration of 31.3 and 15.6 respectively showed a 4, 4.5 and 3-log kill respectively against Neff's cysts within 24 hours which is double the rate seen in the absence of the Lipodisq® carriers at 2-log kill for PHMB and CHLX. The OCT combined with Lipodisq® at a concentration of 3.9 µg/mL gave 3-log reduction compared with a ≈3-log of OCT in its own at higher concentration of 7.8 µg/mL over 24 hours.

This is first time to show that the activity of Lipodisq® carriers combined with biguanide compounds over time kill against cysts of *Acanthamoeba*. PHMB incorporated with nanoliposomes has been shown to have higher antibacterial activity at 10 mm of zone inhibition against *Staphylococcus aureus* and *Escherichia coli* compared with the zone inhibition obtained from PHMB alone at 25 and 21 mm respectively against *S. aureus* and *E. coli* (Ahani *et al.*, 2017). Octenidine was tested against *Staphylococcus epidermidis* and showed more efficient activity at MIC 0.0125% compared with chlorhexidine which showed MIC at a concentration of 0.05% (Chum *et al*., 2018). Recently, the combination of octenidine with lavender essential oil has been investigated against methicillin-resistant *Staphylococcus* aureus ATCC 43300 and the MIC was found to be at a lower concentration of 0.12–0.86 µg/mL (Kwiatkowski *et al.*, 2020).

5.4.3 Corneal penetration observations

The corneal penetration findings of this study have shown that the penetration of CHLX in the absence of Lipodisq® nanoparticle carriers (the initial of exposure concentration to the cornea is 0.02% (v/v) showed 16.3 µg/mL after 1 hour of incubation, while the penetration of CHLX in the presence of the carriers at the same exposure concentration that utilized for CHLX plus carrier decreased to 10.8 µg/mL after the same period of incubation. These results could be explained by the fact that when the combination of CHLX and Lipodisq® solution was heated to 60 °C before the solution was placed in the HPLC mobile phase, the carrier might have been dislocated from the CHLX solution resulting in inadequate penetration of the corneal tissue or could also be a physical constraint associated

with the different structures (the size, polarity of the carrier). Another possible elucidation for this is that extraction method for CHLX plus Lipodisq® carrier was not sufficient and also there was a limitation of the HPLC detected method, as we believe that the nanoparticles Lipodisq® carriers have been used in many studies for a range of applications. Lipodisq® carrier has been measured throughout the skin by using near-infrared chemical imaging (NIRCI) and it has been found that this carrier penetrated into deeper layers of skin over 30 min (Malvern, 2015). A different carrier of eucalyptus oil has been used to deliver hydrophilic drugs into the skin, due to its small size and its ability to speed up the penetration process. It has been shown that when this carrier is combined with 2% (w/v) of CHLX at a concentration of 10% (v/v) in 70% isopropyl alcohol (v/v), the CHLX achieved penetrated of the skin within only 2 min (Karpanen *et al*., 2008).

5.5 Future research work

The hypothesis for penetration study was that the Lipodisq® carrier would be able to increase the delivery of CHLX into the cornea. However, the findings obtained in this study showed that the concentration of CHLX encapsulated with Lipodisq® carrier that entered the cornea was only 10.8 µg/mL from the former exposure concentration of 0.02% (v/v) after 1 hour and the possible explanation is the limitation of the detected method. In future investigations, it might be possible to use a different extraction approach, such as a fluorescent dye, to monitor the penetration of CHLX combined with a Lipodisq® carrier into intact porcine eyes and compare the results with CHLX as a monotherapy. It would be interesting to see the amount of CHLX plus the carrier that penetrates into the cornea, as this combination is potentially promising as a new treatment for AK *in vivo*.

On the other hand, the biguanide compounds, in particular PHMB and chlorhexidine are currently used in the treatment of patients suspected of having *Acanthamoeba* in their eyes. For further studies, it would be important to use a pig or sheep eyes as an *ex vivo* models to analyse and observe the depth of penetration of these compounds within corneal tissue. This essay helps to identify which compound can be more penetrated within a short time inside the cornea and will use these observations as a recommendation for *in vivo* treatment. The reasons for the use of an *ex vivo* pig or sheep models are the simplicity of the

assay which does not require ethical approval and special laboratories to carry out the experiments. Also, I would much prefer to use an *ex vivo* model as it not expensive, accessible, easy to handle and manage. Conversely, there are several disadvantages of using mice, hamster, rabbit as *in vivo* models, including the following: requiring animal ethics approval for *in vivo* research, difficult to handle and needing animal laboratories, and often expensive to order.

5.6 Conclusion

Taken together, the findings of the drug testing in the current study showed that the combination of biguanides with Lipodisq® carriers enhanced the antimicrobial activity ≈ 1 up to 7-fold against cysts and trophozoites of *Acanthamoeba* spp*.* compared with the use of biguanides in their own. Also, complete kill was observed at a 4 and 4.5-log reductions when the PHMB and CHLX were incorporated with Lipodisq® carriers compared with PHMB and CHLX alone, which gave only 2-log reduction. Octenidine alone found to be more active than PHMB and CHLX in their own and these results may support using octenidine as an effective treatment for AK. Furthermore, no toxicity was observed from the combination of biguanide compounds with Lipodisq® carriers when they were tested on human epithelial cell lines and this is a significant indication of the potential use of these formulations for the future treatment of AK. The HPLC analysis of corneal penetration indicated that the Lipodisq® carrier incorporated with CHLX did not enhance the penetration of CHLX into the corneal tissue, as the initial exposure concentration was 0.02% (v/v) and the concentration that penetrated inside the cornea being 10.8 µg/mL after 1 hour of exposure compared with 16.3 µg/mL for CHLX alone, which penetrated more deeply and these findings are related to the extracted method utilized in this study.

Chapter Six

Examining the effect of adrenoceptors and cellulose synthesis inhibitors on the conversion of *Acanthamoeba* trophozoites into the cyst and protocyst form

Chapter 6: Examining the effect of adrenoceptors and cellulose synthesis inhibitors on the conversion of *Acanthamoeba* trophozoites into the cyst and protocyst form

6.1 Introduction

6.1.1 The encystment of *Acanthamoeba*

As stated in chapter 1 section 1.1, *Acanthamoeba* has two distinct stages to its life cycle: the vegetative stage known as the trophozoite, in which it grows, feeds and multiplies and the dormant stage, known as a cyst, that forms when the organism encounters hard environmental conditions such as heat and hyper osmolarity (Byers *et al*., 1991). A number of signals have been found to induce the encystment in *Acanthamoeba* including starvation, elevated pH, osmolarity and the presence of certain drugs (Neff *et al*., 1964). A physical barrier can be produced from the cysts of *Acanthamoeba*, which makes this organism resistant to most of the antimicrobial agents and biocides that used for contact lens disinfection (Turner *et al*., 2000a). When the trophozoites of *Acanthamoeba* exposed to a disinfection containing propylene glycol, it switched to another form termed immature (protocyst) and the process took around 2 hours to complete, unlike cysts which takes around 24 hours to form and so this could be a potential new stage of *Acanthamoeba* (Kilvington *et al*., 2008).

The catecholamines including dopamine, norepinephrine, and epinephrine in mammalian systems are often hormones or neurotransmitters. Epinephrine is a hormone released from the adrenal medulla and is involved in the coordination of physiologic functions including blood pressure, heart rate and levels of circulating glucose (Malven, 1993). There are five different enzymes involved in the synthesis pathway of catecholamines (Figure 6.1). The synthesis begins from the amino acid phenylalanine, which involves the conversion of tyrosine to levodopa (L-DOPA) by the enzyme tyrosine hydroxylase and then decarboxylation of DOPA to dopamine by decarboxylase enzyme. Subsequent step is convert dopamine to norepinephrine by dopamine-β- hydroxylase enzyme and finally the phenylethanolamine-*N*-methyltransferase enzyme methylated the norepinephrine to epinephrine (Goodall and Kirshner, 1958). As can be seen in

Figure 6.1 below, the chemical structure for epinephrine consists of catechol ring and an amine group consisting of a benzene ring with two hydroxyl groups.

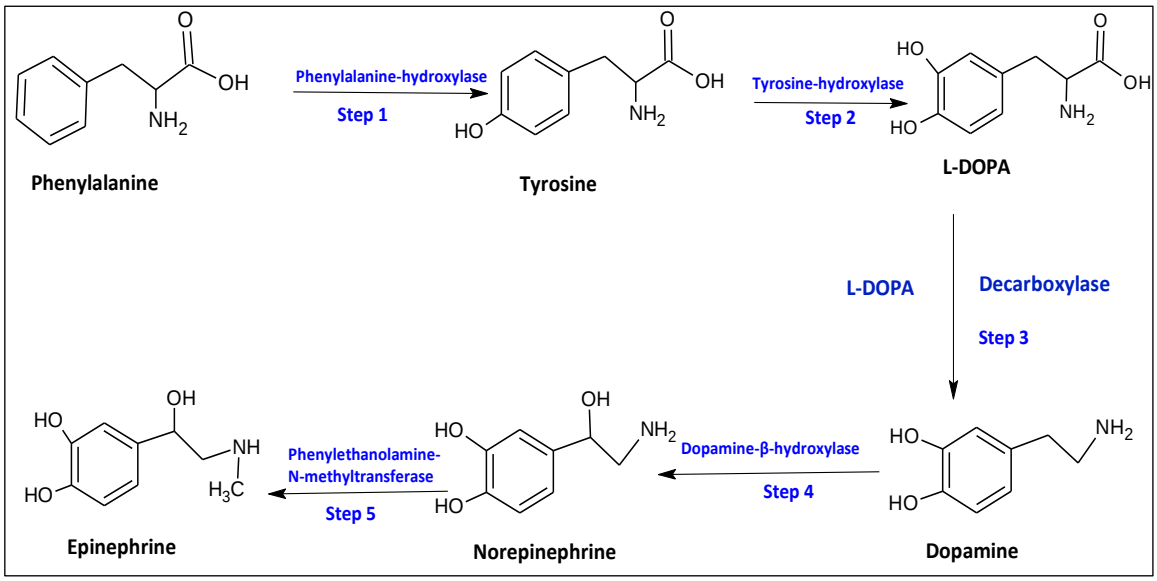

Figure 6-1: Synthesis pathway of epinephrine

6.1.2 G protein coupled receptors and *Acanthamoeba*

G-protein coupled receptors (GPCRs) are seven transmembrane proteins and form the largest, diverse group of mammals (Schöneberg *et al*., 1999). These proteins are encoded by the genomes and can regulate the signalling system. G-proteins can be divided into two major classes, the monomeric, small GTPases and the heterotrimeric G-protein complexes (Oldham and Hamm, 2008, LeVine, 1999). The G- proteins that are associated with GPCRs are heterotrimeric, meaning they have three different subunits, Gα, Gβ, and Gγ, two of which, Gβ and Gγ, are attached to the plasma membrane by lipid anchors as illustrated in Figure 5.2 (Morris and Malbon, 1999). G-proteins in normal state, the Gα subunit is bound to the GDP and this complex is bound with high affinity to the heterodimer segment Gβγ (Morris and Malbon, 1999). Once the receptor (GPCR) is activated, GDP is released from Gα allowing binding GTP as an alternative (Oldham and Hamm, 2008). GPCRs play a crucial role in mammalian cells by regulating a number of cellular functions, such as cell cycle, proliferation and cytoskeletal rearrangements (Wennerberg and Der, 2004, Clarke *et al*., 2013).

Also, in human GPCRs regulate numerous physiological activities included metabolism, secretion, growth, inflammatory, and immune responses (Chattopadhyay, 2014). In a study conducted by Clarke *et al*. (2013) found that *Acanthamoeba* genome encodes 35 G protein couple receptors, which act as sensors for extracellular inducements and consequently represent prospective candidates for the initial step in signaling at the starting of encystment in *Acanthamoeba*.

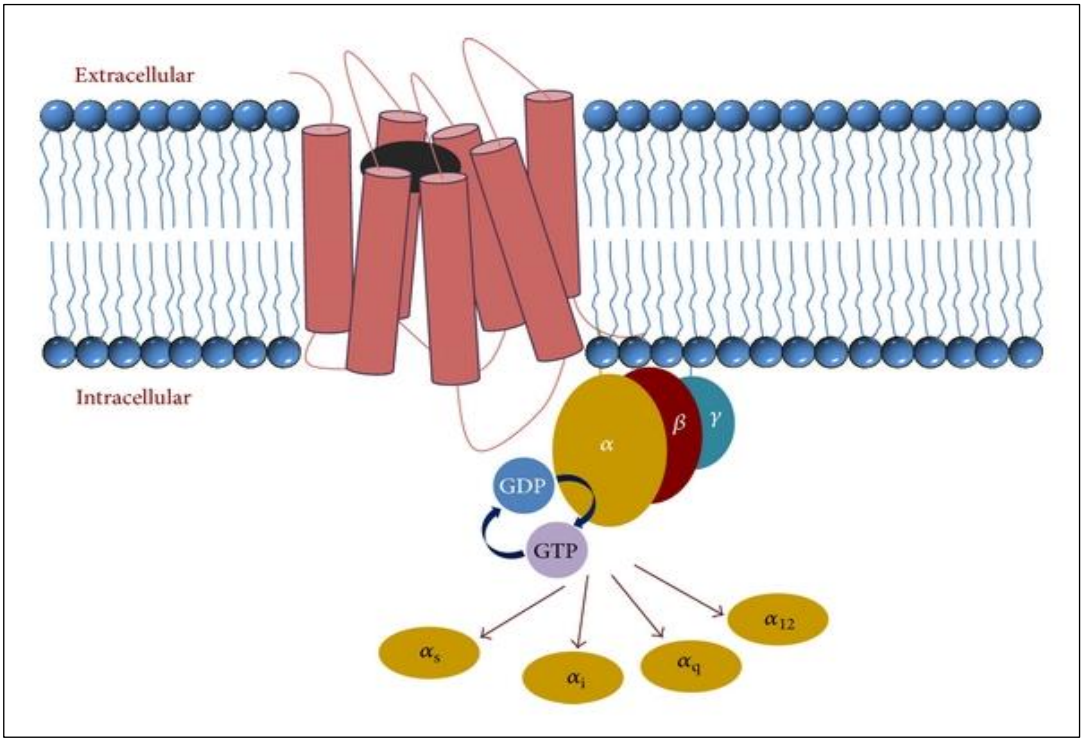

Figure 6-2: A diagram showing the seven transmembrane proteins of G-protein coupled receptors (GPCRs) and these proteins convert extracellular signals to inside the cells. There are a variety of ligands that can stimulated the GPCRs included biogenic amines, amino acids, ions, lipids, peptides, and several exogenous ligands for instance pheromones. As can be seen in this diagram the ligand (in black colour) binds at the transmembrane region of the GPCRs. Activation of heterotrimeric G proteins α, β, and γ subunits is related to the binding of particular ligands to the GPCRs. The other subunits of G proteins involving αs, αi, αq, and α_{12} play an important role in regulating the signaling cascades. The image taken from (Chattopadhyay, 2014).

The first messenger system contains extracellular factors, often hormones or neurotransmitters, such as epinephrine, growth hormones. These first messengers may not physically cross the phospholipid bilayer to initiate changes within the cell in the mammalian system and because of this functional limitation (Lodish *et al*., 2000), the transduction mechanisms transduce from the first messenger system to the second messengers (Lodish *et al*., 2000). The second messengers are small molecules such as cyclic AMP (cAMP), which are initiate intracellular signalling pathways (Lodish *et al*., 2000), and these molecules alternate signals that received by the receptors on the surface of the cell and the activation of single G-protein leads to the production of thousands of second messenger molecules. Furthermore, G-protein and its downstream signalling molecules such as Rab GTPases, Rho and Ras, are involved in the pathogenesis of *Entamoeba histolytica* and they have been highlighted as possible targets for pharmacological manipulation (Bosch and Siderovski, 2013).

Epinephrine plays a critical role in targeting the encystment of *Acanthamoeba* and the process is probably mediated by a receptor that stimulates cyclic AMP (cAMP) synthesis (Srivastava and Shukla, 1983, Krishna, 1975). In a number of eukaryotic cells in mammalian systems cyclic cAMP dependant signalling pathways play an important role in regulating three important mechanisms involved in cell growth, metabolism and differentiation (Abel *et al*., 2001). *Acanthamoeba* shares a similarity with the mammalian in response to the action of the endogenous epinephrine, it was observed that in mammalian system the activity for glycogen phosphorylase is increased and resulted in breakdown of the livers glycogen stores (Riley and Haynes, 1963). Likewise, the breaking down of glycogen into glucose monomers by glycogen phosphorylase enzyme is one of the most important activities that is detected during the encystment of *Acanthamoeba* (Weisman *et al*., 1970).

6.1.3 Production of cellulose in *Acanthamoeba*

Acanthamoeba cyst walls are mostly made up of cellulose polymer. A recent study by Garajová *et al*. (2019) used a variety of techniques, included light, confocal laser scanning and transmission electron microscopy, to investigate the elements of cysts. They found that cellulose fibrils exist in the endocyst and ectocyst layers of the *Acanthamoeba* cyst wall. This observation is supported by

a previous study by Chavez-Munguia *et al*. (2005) which reported that cellulose polymers are found in both layers of the *Acanthamoeba* cyst wall. The synthesis of cellulose polymers during the encystment of *Acanthamoeba* is initiated by the breakdown of glycogen in the trophozoites into glucose-1-phosphate caused by glycogen phosphorylase (Greenberg *et al*., 2006). There are four enzymatic steps involved in the synthesis of cellulose polymers from glucose in *Acanthamoeba*: the first step is the phosphorylation of glucose into glucose-6-phosphate by hexokinase and then the isomerisation of glucose-6-phosphate to glucose-1-phosphate by phosphoglucomutase. The next step is the synthesis of UDP-glucose by UDP-glucose pyrophosphorylase and the final step is the formatting of cellulose by cellulose synthase as shown in Figure 6.3 (Moon and Kong, 2012).

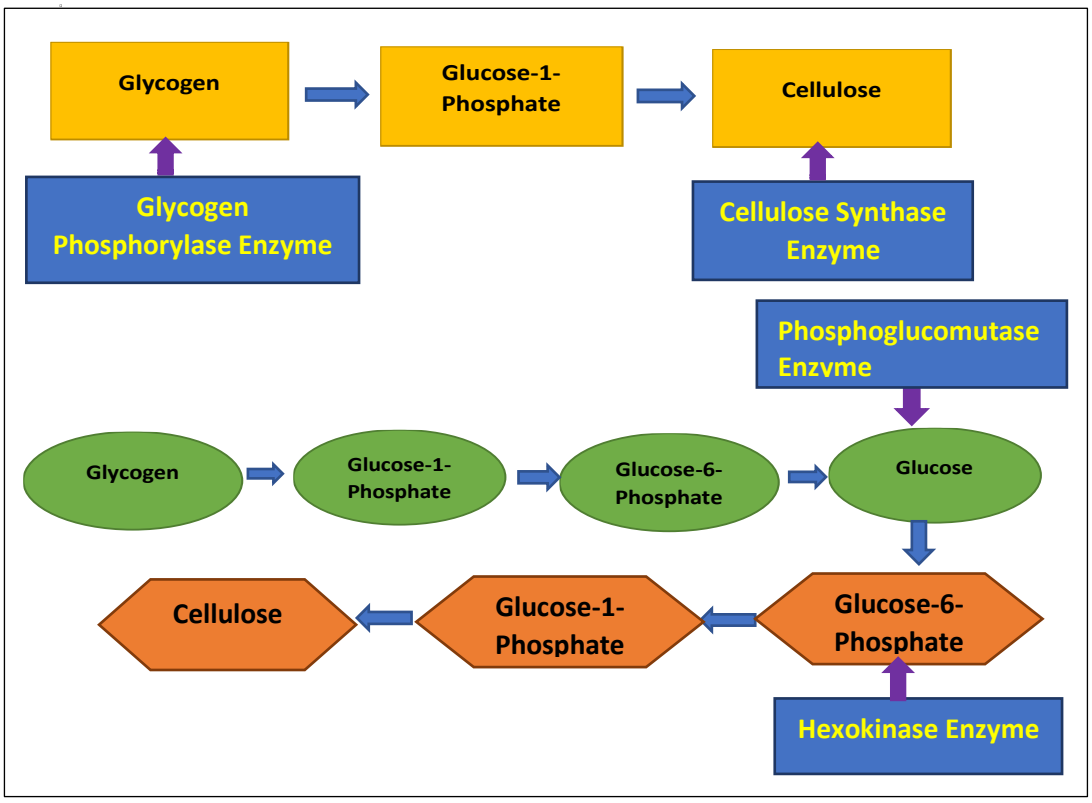

Figure 6-3: Diagram showing the cellulose biosynthesis pathway in *Acanthamoeba*. The process begins with the breakdown of the glycogen in the trophozoites when *Acanthamoeba* triggers to encyst into glucose. The biosynthesis of cellulose in the cyst wall of *Acanthamoeba* occurs through several steps, as can be seen in the figure. Adapted from (Moon and Kong, 2012).

6.1.4 Aims and objectives of this chapter

The first aim of this chapter is to investigate the transformation of *Acanthamoeba* trophozoites into cysts and protocysts in the presence and absence of a variety of adrenoreceptors, including agonists and antagonists. The second aim is to examine the effect of cellulose synthesis inhibitors (CSI) including isoxaben and 2,6-dichlorobenzilnitrate (DCB) on their own and in combination on the formation of *Acanthamoeba* cysts and protocysts. This chapter provides a more detailed investigation regarding the effects of CSI on the transformation of trophozoites into cysts and protocysts in *Acanthamoeba*. It is known that the cyst walls of *Acanthamoeba* are partially made of cellulose, so this study investigated the ability of these cellulose synthesis inhibiting herbicides to block the encystment process and also inhibit the protocyst formation of *Acanthamoeba*. The mechanism of action for DCB and Isoxaben is interfering and blocking the cellulose synthase enzyme during cellulose biosynthesis in *Acanthamoeba*. The present work is an extension of the work reported earlier by (Heaselgrave and Kilvington, 2016) and this study is different from the aforementioned study, as the current research attempted to examine the effect of the β ultra-long agonist indacaterol in order to target the receptor in the trophozoites membrane of *Acanthamoeba*. The agonist indacaterol can bind to the receptor and will not diffuse compared with β_2 agonist salbutamol which can bind to the receptor for a short period. Also, to examine different antagonists included levobunolol and betaxolol against the conversion of trophozoites into cysts and protocysts alongside the other antagonists which tested previously against cyst formation of *Acanthamoeba*. The objectives of this chapter are stated below:

I. Test adrenoceptor agonists and antagonists against the transformation of trophozoites into cysts and protocysts in the presence and absence of epinephrine.
II. Test adrenoceptor antagonists against the conversion of trophozoites into protocysts in the presence of epinephrine.
III. Evaluate the effect of isoxaben and DCB on the transformation of trophozoites into cysts and protocysts, as these compounds inhibit the cellulose synthase enzyme in *Acanthamoeba*.
IV. Investigate the effect of DCB and isoxaben in their own and in combination on the cysts and protocysts formation.

6.2 Methods

6.2.1 Preparation of organisms

Acanthamoeba castallanii (ATCC 30868) was cultured and maintained as detailed in chapter 2, section 2.3. *Acanthamoeba castallanii* (ATCC 30868) was selected for the encystment investigations among *Acanthamoeba castallanii* (ATCC 50370) and *Acanthamoeba polyphaga* (ATCC 30461) as it has more favourable encystment profile in Neff's medium and provided natural encystment levels. In contrast, the other strains, *Acanthamoeba castallanii* (ATCC 50370) and *Acanthamoeba polyphaga* (ATCC 30461), encysted rapidly and it was difficult to distinguish between the normal encystment process and encystment in the presence of the agonists or antagonists. *Acanthamoeba castallanii* (ATCC 50370) was used for the production of protocysts as this strain provided natural protocyst formation.

6.2.2 Preparation of adrenoreceptor agonists and antagonists

The agonists and antagonists were prepared and dissolved in appropriate solvent at different concentrations in order to be tested against the transformation of trophozoites of *A. castallanii* (ATCC 30868) into cyst or protocyst forms (Tables 6.1 & 6.2).

Table 6-1: The agonists used in this study for the encystment assay

Name	Specificity	Supplier	Chemical Name
Dobutamine	B_1 agonist	VWR, (Lutterworth, U.K)	(±)-3,4-Dihydroxy-N-[3-(4-hydroxyphenyl)-1-methylpropyl]-β-phenethylamine hydrochloride
Epinephrine	α/β agonist	Sigma, (Poole, U.K.)	L-3,4-Dihydroxy-α-(methylaminomethyl)benzyl alcohol D-hydrogen bitartrate salt
Isoprenaline	β_1/β_2 agonist	Sigma, (Poole, U.K.)	1-(3′,4′-Dihydroxyphenyl)-2-isopropylaminoethanol hydrochloride
Salbutamol	β_2 agonist	Sigma, (Poole, U.K.)	α-[(tert-Butylamino)methyl]-4-hydroxy-m-xylene-α,α′-diol
Clenbuterol	β_2 agonist	VWR, (Lutterworth, U.K)	4-Amino-α-(t-butylaminomethyl)-3,5-dichlorobenzyl alcohol hydrochloride
Indacaterol	β agonist	Sigma, (Poole, U.K.)	5-[(1R)-2-[(5,6-diethyl-2,3-dihydro-1H-inden-2-yl)amino]-1-hydroxyethyl]-8-hydroxy-1H-quinolin-2-one

Table 6-2: The antagonists used in this study for the encystment assay

Name	Specificity	Supplier	Chemical Name
Betaxolol	β_1 antagonist	Moorfields Eye Hospital (London U.K.)	1-[4-[2 (Cyclopropylmethoxy)ethoxy]phenoxy]-3-(propan-2-ylamino)propan-2-ol
Levobunolol	β_2 antagonist	Moorfields Eye Hospital (London U.K.)	(−)-5-[3-(tert-Butylamino)-2-hydroxypropoxy]-3,4-dihydronaphthalene-1(2H)-one hydrochloride
Propranolol	β_2 antagonist	Sigma, (Poole, U.K.)	(±)-1-Isopropylamino-3-(1-naphthyloxy)-2-propanol hydrochloride
Metoprolol	β_1 antagonist	Sigma, (Poole, U.K.)	(±)1-(Isopropylamino)-3-[p-(β-methoxyethyl)phenoxy]-2-propanol (+)-tartrate salt
Butaxamine	β_2 antagonist	VWR, (Lutterworth, U.K)	α-(1-[t-Butylamino]ethyl)-2,5-dimethoxybenzyl alcohol

6.2.3 *Acanthamoeba* encystment assay

The encystment experiments were performed in polypropylene (50 mL) tubes. Initially, 5 mL of AC#6 growth medium was added to each tube and trophozoites at a density of 2.5 x 10^5 cells/mL were added at time 0 to each tube. Then, different concentrations of agonists and antagonists and the required concentration of $MgCl_2$ were applied. Subsequently, the tubes were incubated in an orbital shaking incubator at 30 °C and 120 rpm for 7 days. The number of cysts were determined using a Modified Fuchs Rosenthal haemocytometer count. To count the number of cysts, an aliquot of the test solution in triplicate was used, to which was added 20 µL of 0.1% (v/v) N-lauryl sarcosinate Sarkoysl in order to aid the counting of the cells under an inverted microscopy.

6.2.4 *Acanthamoeba* protocyst assay

The protocyst experiments were carried out also in polypropylene (50 mL) tubes. 5 mL of Neff's medium plus 0.5% (v/v) of propylene glycol was added to each tube and trophozoites at a density of 2.5 x 10^5 cells/mL were added at time 0. Agonists and antagonists were added to the corresponding tubes and then the tubes were incubated in an orbital shaking incubator at 30 °C and 120 rpm for 24 hours. The protocyst count was performed in triplicate by removing an aliquot of

the test solution and the counting was performed using a haemocytometer under an inverted microscopy.

6.2.5 Effect of DCB and isoxaben on the conversion of trophozoites into cysts assay

In order to determine the effect of cellulose synthesis inhibitors 2,6-dichlorobenzonitrile (DCB) and isoxaben on the *Acanthamoeba* encystment process, a method adapted from Moon *et al*. (2015) was used in this study. DCB and isoxaben were dissolved in methanol at a concentration of 1 mM and then serially diluted to give a range of 500-50 µM. The assay was performed in 12-well plates containing 3 mL of Neff's encystment medium per well (as presented in Figure 6.4). The wells were seeded with 2.5 x 10^5 cells/mL of *Acanthamoeba castellanii* (ATCC 50370) trophozoites. Several concentrations of DCB and isoxaben, ranging from 50 µM to 500 µM, were added to the wells and the plates were incubated at 30°C for 72 hours. After incubation, the cells were enumerated to determine the number of cysts. This was achieved by removing an aliquot of the test solution in triplicate to which was added 20 uL of 0.1% (v/v) N-lauryl sarcosinate (Sarkoysl) as used for encystment study which detailed in section 6.2.3.

6.2.6 Effect of DCB and isoxaben on the transformation of trophozoites into protocysts assay

This assay was performed to examine the effect of DCB and isoxaben on the formation of protocysts of *Acanthamoeba castellanii* (ATCC 50370). The method detailed in section 5.2.5, modified from Moon *et al*. (2015), was used. Again, to each well, 30 µL of 2.5 x 10^5 cells/mL trophozoites of *A. castellanii* (ATCC 50370) were seeded in 12-well plates containing 3 mL of Neff's + 0.5% (v/v) propylene glycol and incubated for 24 hours. Similarly, various concentrations of DCB and isoxaben were tested and the protocyst count was performed in triplicate using a haemocytometer under an inverted microscopy.

Figure 6-4: The 12-well plate used for cellulose synthesis inhibitor assays

6.3 Results

The results in this chapter are focused on the effect of different adrenoreceptors agonists and antagonists and cellulose synthesis inhibitors including 2,6-dichlorobenzonitrile (DCB) and isoxaben on the encystment process of *Acanthamoeba* and protocysts formation.

6.3.1 The effect of agonists on the cysts formation of *Acanthamoeba*

Different agonists were tested in this study to see if they were able to prevent or block the encystment of *Acanthamoeba castellanii* (ATCC 30868) in AC#6 growth medium supplemented with 50 mM $MgCl_2$. A higher level of encystment was observed with 0.5 mM of epinephrine at 90.3%. A lower concentration of indacaterol at 100 µM also demonstrated high encystment level of 85.7% and the percentage of encystment decreased slightly with 100 µM of dobutamine at 83.5%. Using One Way ANOVA analysis indicated that the concentrations of 0.5 mM of epinephrine and 100 µM of indacaterol and dobutamine were statistically significant ($P < 0.001$) compared to 50 mM of $MgCl_2$ or AC#6 medium as a controls. Only a concentration of 10 µM of salbutamol was tested in this study, due to when the concentration was increased the salbutamol caused huge lysis of the *Acanthamoeba* trophozoites, and the concentration of 10 µM resulted in a blocking of the encystment process at 36.8%. One Way ANOVA analysis showed

that salbutamol at a concentration of 10 µM was significant (P ≤ 0.001) compared to the AC#6 medium as a control but no significant was observed from the tested the same concentration of salbutamol in relation to the $MgCl_2$ as a control. Isoprenaline and clenbuterol at a concentration of 1 mM showed encystment at 75.4% and 60% respectively (Figure 6.5). Additional statistical analysis of two sample t-test for the means of all the agonists examined relative to the controls demonstrated p-value at p=0.0003.

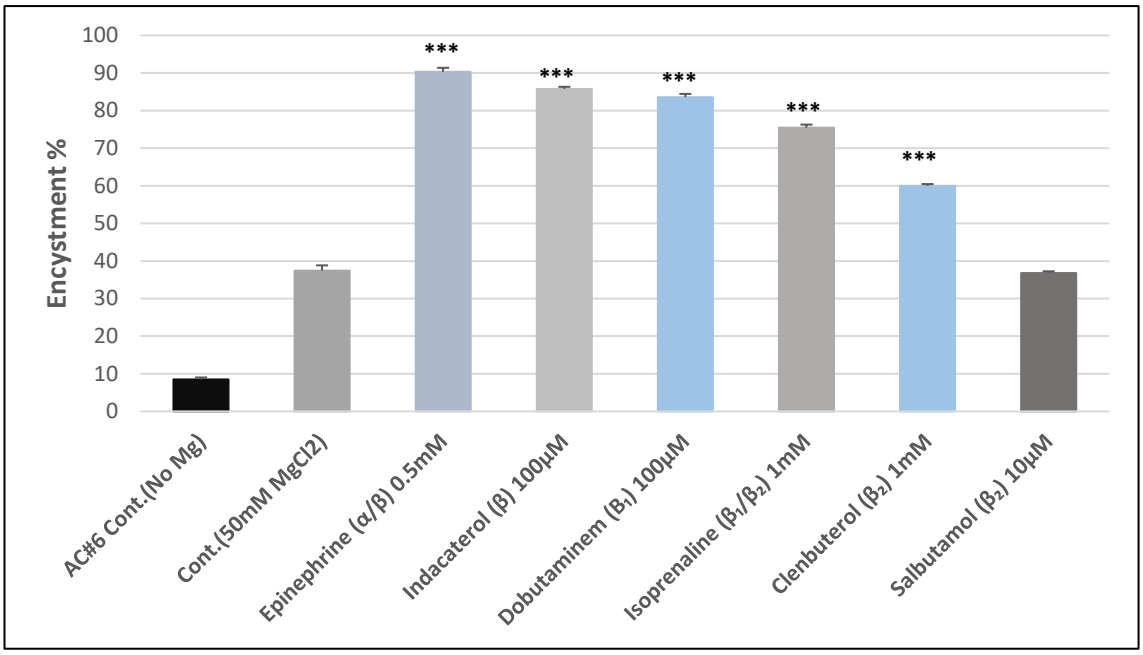

Figure 6-5: The effect of different agonists on the encystment of *Acanthamoeba castellanii* (ATCC 30868) in AC#6 growth medium. The standard error of the mean (SEM) represented the error bars (n=3). One-way analysis of variance (ANOVA) was performed. Asterisks represent values statistically significant (***P<0.001) between the tested agonists and the AC#6 medium or 50 mM of $MgCl_2$ as controls.

6.3.2 The effect of antagonists on the cysts formation of *A. castellanii*

The results obtained from the antagonists are presented in Figure 6.6. Levobunolol at a concentration of 500 µM induced the encystment at 53.7% and at the same concentration, a small decrease in the level of the encystment was observed with both butaxamine and propranolol, at 46.2% and 43.9% respectively. A concentration 500 µM of all the antagonists tested in this study

was significant ($P < 0.001$) compared to that of the $MgCl_2$ or AC#6 medium as a controls. Also, two sample t-test for the mean of all the antagonists evaluated compared to the controls given p-value at p=0.002 and this is statistically significant. Clear inhibition of the encystment process was found with metoprolol and betaxolol at concentrations of 500 µM exhibiting only 14.6% and 22.3 % of encystment respectively.

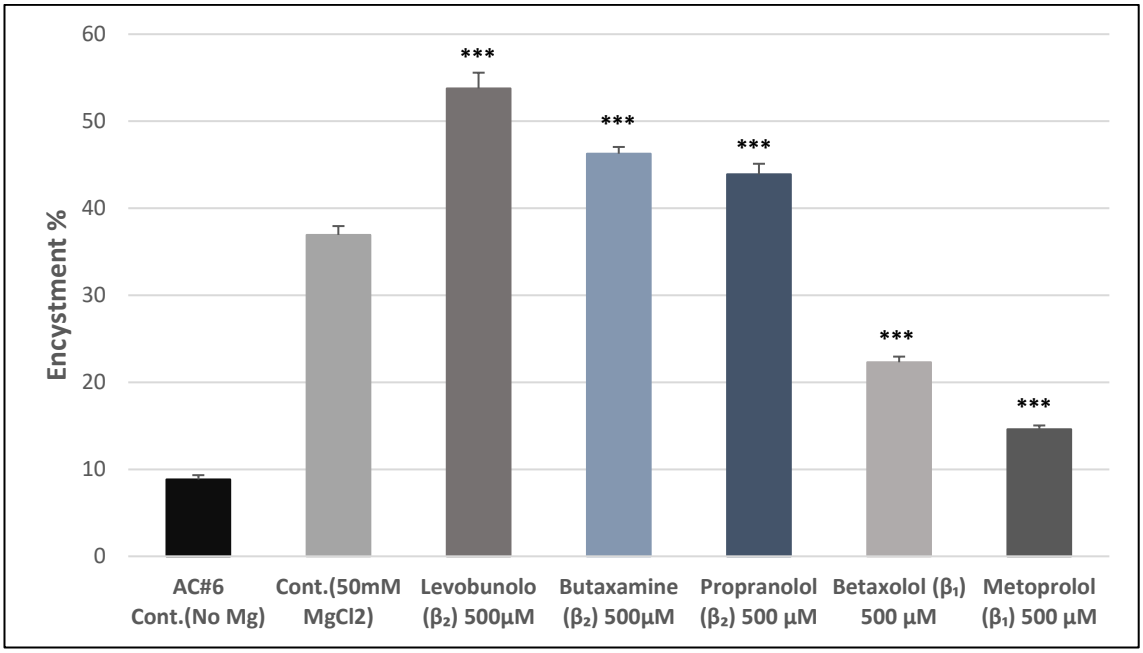

Figure 6-6: The effect of antagonists on the encystment of *Acanthamoeba castellanii* (ATCC 30868) in AC#6 growth medium. The antagonists tested to block endogenous of catecholamines in *Acanthamoeba*. The standard error of the mean (SEM) represented the error bars (n=3). One-way analysis of variance (ANOVA) was performed. Asterisks represent values statistically significant (***P<0.001) between the tested antagonists and the AC#6 medium and 50 mM of $MgCl_2$ as controls.

The experiment was repeated with the same antagonists but in the presence of 500 µM of epinephrine to check the effect of these antagonists on the encystment of *Acanthamoeba*. Epinephrine was tested alone, and it led to a higher level of encystment at 87.8% and combined with β_1 metoprolol at a concentration of 500 µM showed the lowest level of the encystment at 33.3%. Also, a higher rate of encystment was observed with indacaterol in its own at a concentration of 100 µM gave 85.7% encystment and when this agonist mixed with 100 µM of β_1 metoprolol the encystment level was reduced down to 37.6%. Propranolol at a

concentration of 500 µM combined with epinephrine was exhibited 64.8% of encystment. The next antagonist that was combined with epinephrine at a concentration of 500 µM was β_2 levobunolol and the percentage of encystment decreased to 62.5 %. Of the antagonists that were tested in the current study, β_1 betaxolol and β_2 butaxamine, at the same concentration of 500 µM combined with epinephrine, giving 48.7% and 52.3% encystment respectively. Furthermore, all the antagonists that were mixed with epinephrine at a concentration of 500 µM promoted the encystment process ($P < 0.001$) compared with $MgCl_2$ or AC#6 medium as a controls, excepted the metoprolol which had biggest reduction on the encystment compared to the $MgCl_2$ as a control (Figure 6.7). Moreover, two sample t-test for all antagonists combined with epinephrine in comparison to the controls displayed p-value at p=0.0001.

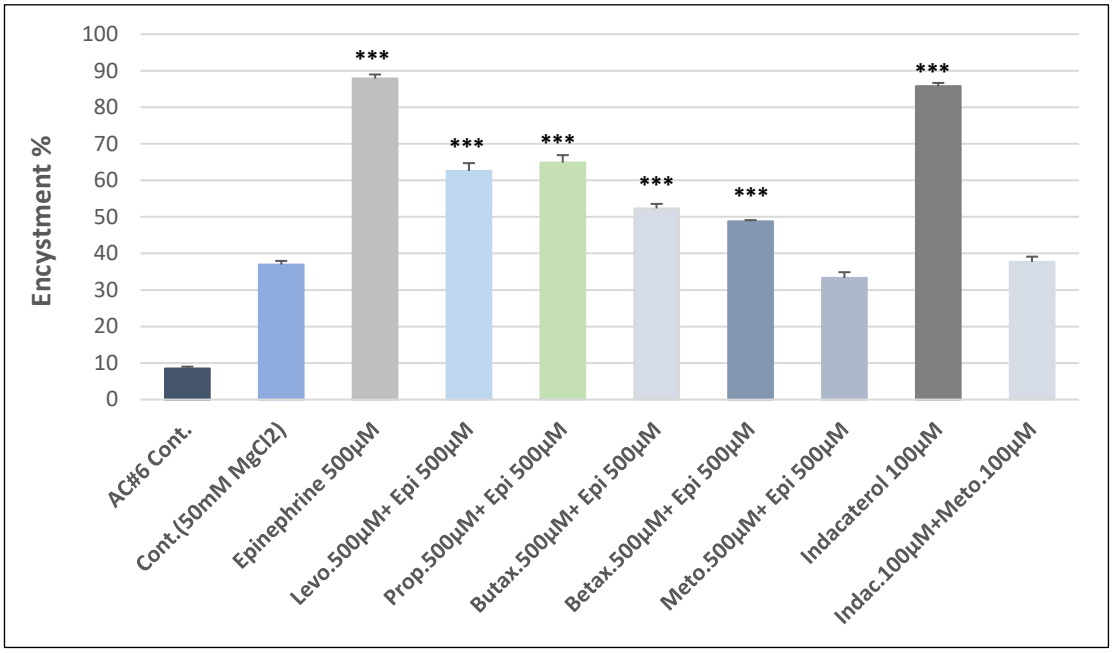

Figure 6-7: The effect of antagonists in the presence of epinephrine on the encystment of *Acanthamoeba castellanii* (ATCC 30868) in AC#6 growth medium. The epinephrine combined with the antagonists to block endogenous & exogenous of catecholamines in *Acanthamoeba*. The standard error of the mean (SEM) represented the error bars (n=3). One way analysis showed of variance (ANOVA) was performed. Asterisks represent values statistically significant (***P<0.001) between the tested antagonists plus epinephrine and the AC#6 medium or 50 mM of $MgCl_2$ as controls.

6.3.3 The effect of antagonists on the protocysts formation of *Acanthamoeba*

Further investigations were performed with the antagonists to assess their effect on the transformation of trophozoites into the protocyst form of *Acanthamoeba castellanii* (ATCC 50370). In this study, levobunolol at a concentration of 500 µM gave the highest percentage of protocyst production at 62.6%. Betaxolol at the same concentration gave 52.7%. However, the other three antagonists that were tested in the current study, metoprolol, butaxamine and propranolol, at a concentration of 500 µM prevented the development of trophozoites into protocysts at 22.9%, 26.6% and 27.3% respectively. These findings showed that different antagonists at a concentration of 500 µM were significant ($P < 0.001$) compared with $MgCl_2$ or AC#6 medium as a controls (Figure 6.8). In addition, for all assessed antagonists, two sample t-test of the mean proved p-value at p=0.004.

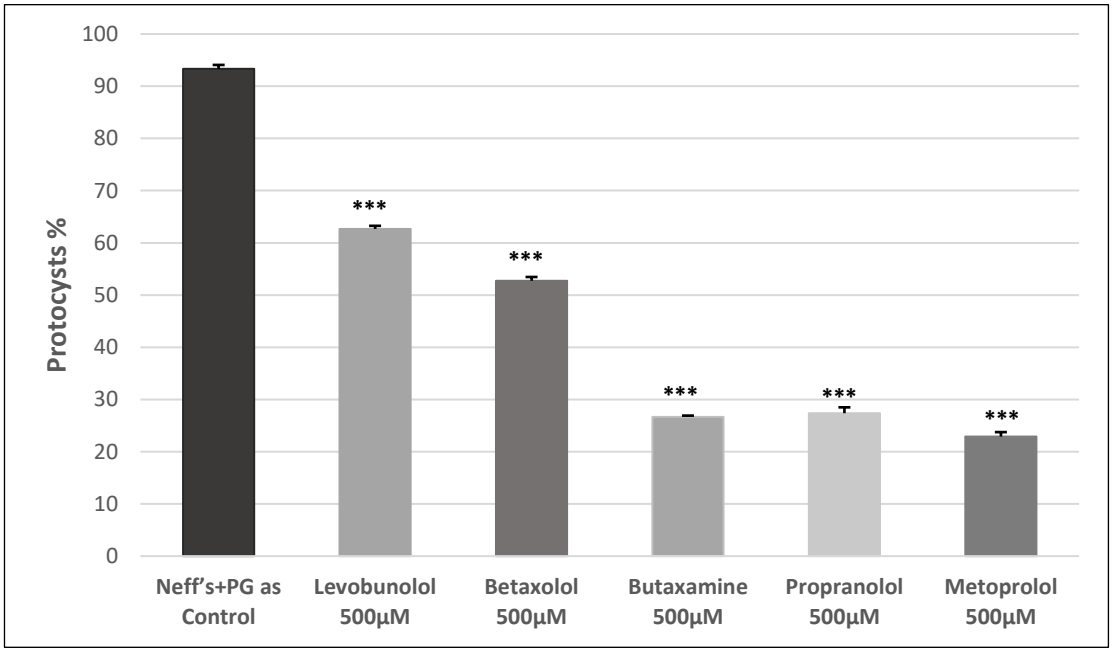

Figure 6-8: The effect of different antagonists on the conversion of trophozoites into protocysts of *A. castellanii* (ATCC 50370) in Neff's medium plus 0.5% (v/v) propylene glycol (PG). The antagonists tested to block endogenous of catecholamines in *Acanthamoeba*. The standard error of the mean (SEM) represented the error bars (n=3). One-way analysis of variance (ANOVA) was performed. Asterisks represent values statistically significant (***P<0.001) between the tested antagonists plus and Neff's-PG medium as control.

As can be seen from the data in Figure 6.9, the protocysts production was increased in the presence of epinephrine. Higher percentages of protocysts were observed with levobunolol and betaxolol at a concentration of 500 µM, giving 69.7% and 57.7%, respectively. Conversely, the other antagonists, propranolol, butaxamine and metoprolol, combined with epinephrine at a concentration of 500 µM prevented protocyst formation, giving figures of 37.4%, 35.6% and 30.9% respectively. One Way ANOVA statistical analysis showed a significant correlation ($P < 0.001$) for all antagonists that were tested in this experiment relative to the $MgCl_2$ or AC#6 medium as a control. Also, two sample t-test analysis of the examined antagonists revealed p-value at p=0.003.

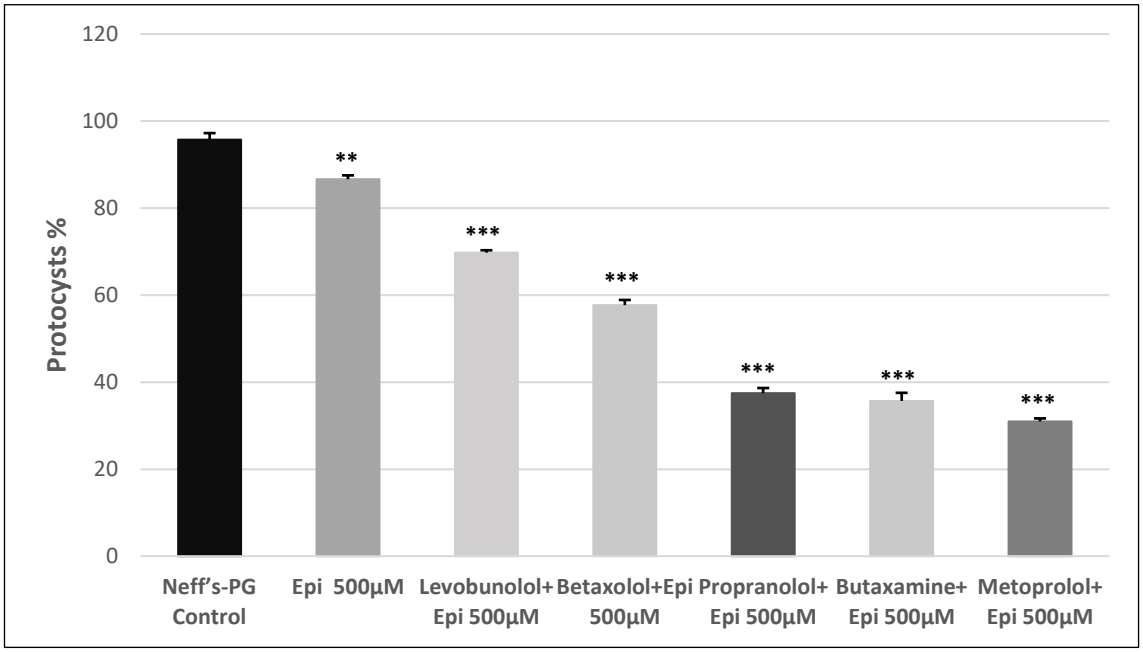

Figure 6-9: The effect of several antagonists in the presence of epinephrine on the conversion of trophozoites into protocysts of *A. castellanii* (ATCC 50370) in Neff's medium plus 0.5% (v/v) propylene glycol (PG). The epinephrine combined with the antagonists to block endogenous & exogenous of catecholamines in *Acanthamoeba*. The standard error of the mean (SEM) represented the error bars (n=3). One-way analysis of variance (ANOVA) was performed. Asterisks represent values statistically significant (***P<0.001) between the tested antagonists plus epinephrine and the Neff's-PG medium as control.

6.3.4 The effect of 2,6-dichlorobenzonitrile (DCB) and isoxaben on the encystment of *Acanthamoeba*

Four different concentrations of 2,6-dichlorobenzonitrile (DCB) and isoxaben (Isox) were evaluated to observe the inhibitory effect of these cellulose synthesis inhibitors on the transformation of trophozoites into the cyst stage.

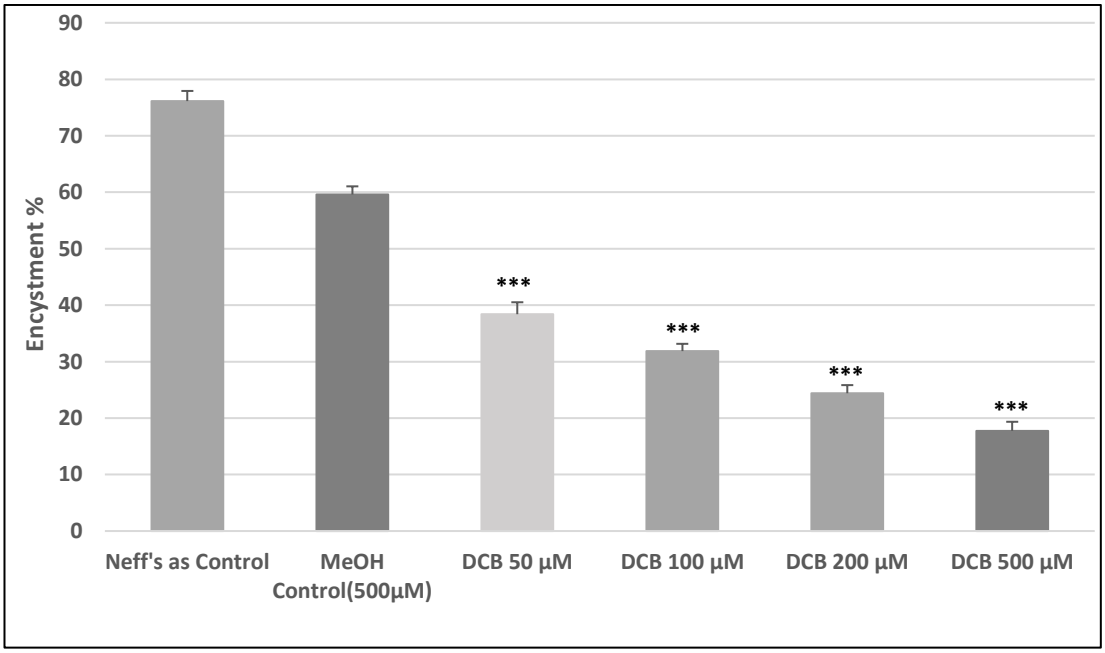

Figure 6-10: The effect of (2,6-dichlorobenzonitrile) DCB on the encystment of *A. castellanii* (ATCC 30868) in Neff's medium. The haemocytometer counts for cysts were performed after 3 days of incubation. The standard error of the mean (SEM) represented the bars (n=3). One-way analysis of variance (ANOVA) was performed. Asterisks represent values statistically significant (***P<0.001) between the tested concentrations of DCB and the Neff's medium or 500 uM MeOH as controls.

When tested against trophozoites, DCB at concentrations of 50 µM, 100 µM and 200 µM prevented the encystment at 38.4%, 31.8% and 24.4% respectively. The encystment level that observed at these concentrations, compared to that of the Neff's or 500 µM of methanol as controls, was statistically significant according to the One-Way ANOVA analysis. A higher concentration of DCB at 500 µM was blocked the encystment of *Acanthamoeba* at 17.7% and the One-Way ANOVA analysis of the concentration of 500 µM of DCB showed significance ($P < 0.001$) compared with the Neff's or methanol at concentration of 500 µM as controls

(Figure 6.10). Two sample t-test analysis was undertaken for the different tested contractions of DCB in relation to the controls showed p-value at p=0.004.

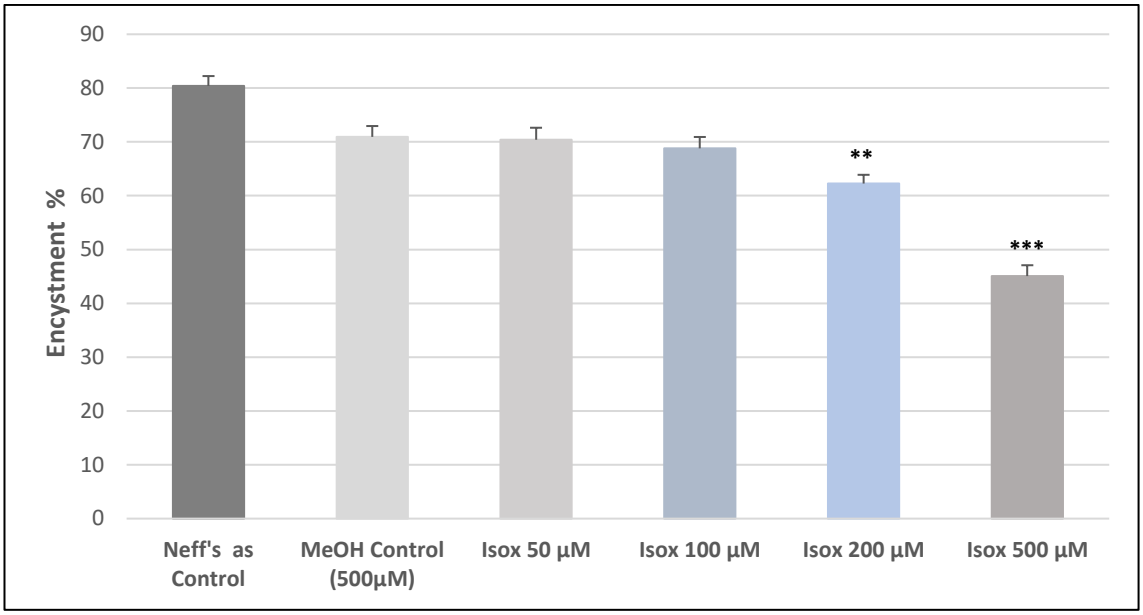

Figure 6-11: The effect of isoxaben on the encystment of *A castellanii* (ATCC 30868) in Neff's medium. The haemocytometer counts for cysts were performed after 3 days of incubation. The standard error of the mean (SEM) represented the error bars (n=3). One-way analysis of variance (ANOVA) was performed. Asterisks represent values statistically significant (***P<0.001) between the tested concentrations of isoxaben and the Neff's medium or 500 uM MeOH as controls.

The higher concentration of isoxaben of 500 µM showed a 45% encystment of *Acanthamoeba,* which is significant (P < 0.001) compared with the Neff's or methanol at concentration of 500 µM as controls. The results revealed that isoxaben concentrations of 50 µM, 100 µM and 200 µM showed relatively similar levels of encystment at 70.4%, 68.8% and 62.2% respectively. Furthermore, when isoxaben was correlated with the methanol control, the effect was not significant as the p-value of t-test showed p=0.07, but at these concentrations of isoxaben, the effects were statistically significant compared with the Neff's as control with p-value at p=0.0002 (Figure 6.11).

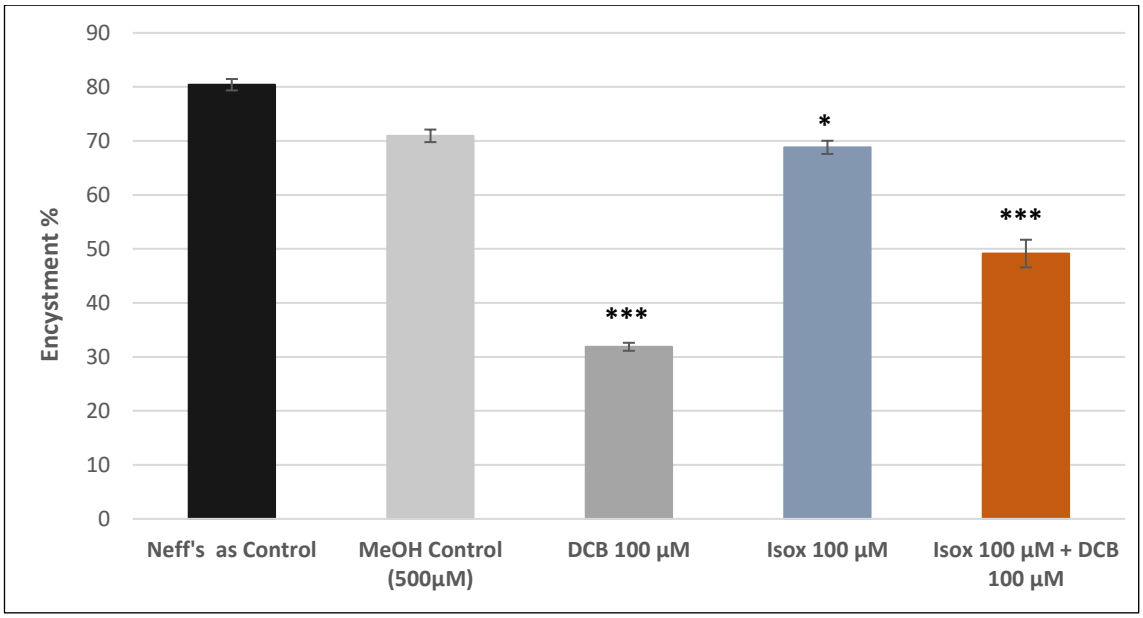

Figure 6-12: The effect of DCB & isoxaben alone and in combination on the encystment of *A castellanii* (ATCC 30868) in Neff's medium. The haemocytometer counts for cysts were performed after 3 days of incubation. The standard error of the mean (SEM) represented the error bars (n=3). One-way analysis of variance (ANOVA) was performed. Asterisks represent values statistically significant (***P<0.001) between the tested concentrations of DCB and the Neff's medium or 500 uM MeOH as controls.

More testing was carried out to examine the effect of DCB and isoxaben individually and in combination at a concentration of 100 µM on the conversion of trophozoites into cysts. Based on the findings that obtained from this experiment, the DCB alone at a concentration of 100 uM gave encystment level at 31.8% and much higher rate of encystment was occurred at 68.8% for isoxaben at a concentration of 100 uM. However, when 100 uM of isoxaben was added to the same concentration of DCB and tested, an antagonistic impact was observed as the transformation of trophozoites into cysts was found to be at rate of 49.1% as presented in Figure 6.12. Two sample t-test analysis for the of DCB in its own and in combination with isoxaben was found to be significant compared to the controls, as the p-value was recorded at p=0.0049.

6.3.5 The effect of DCB and isoxaben on the transformation of trophozoites into protocysts

The effect of the same compounds, DCB and isoxaben on the conversion of trophozoites into protocysts was also examined.

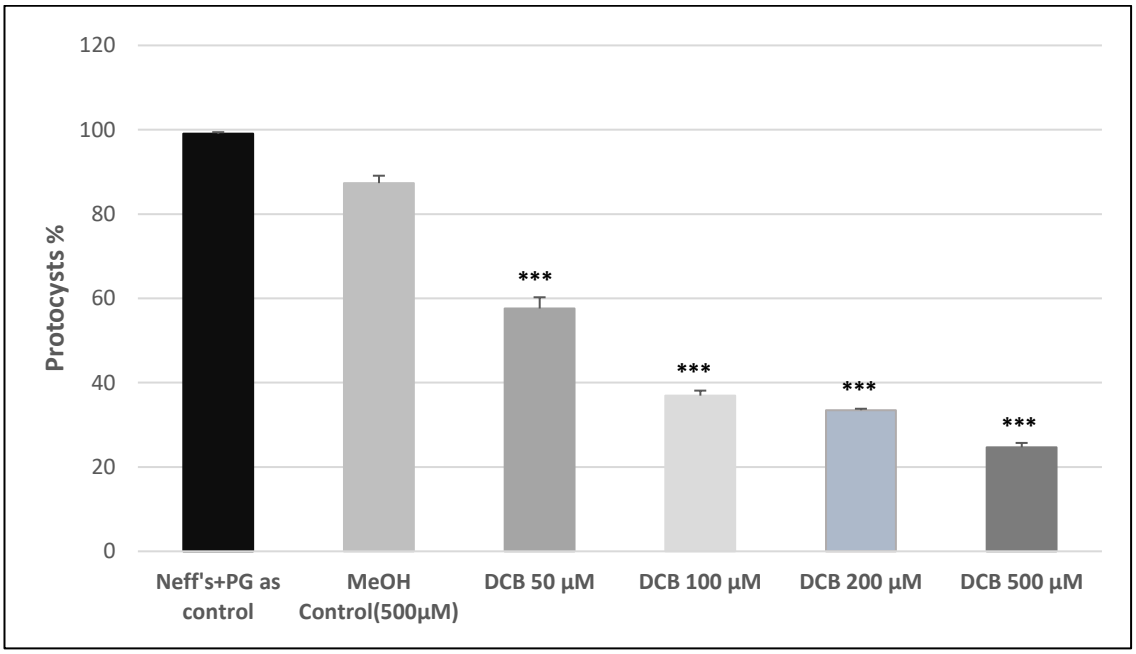

Figure 6-13: The effect of DCB on the transformation of trophozoites of *A. castellanii* (ATCC 50370) into protocysts in Neff's medium plus 0.5% (v/v) propylene glycol. The haemocytometer count for protocysts was performed after 24 hours of incubation. The standard error of the mean (SEM) represented the error bars (n=3). One-way analysis of variance (ANOVA) was performed. Asterisks represent values statistically significant (***P<0.001) between the tested concentrations of DCB and the Neff's-PG or 500 uM MeOH as controls.

It can be seen from the results in Figure 6.13 that there is a clear increase in the inhibition of protocysts. The higher concentration of DCB at 500 µM exhibited a 24.6% of protocysts and this value was significant ($P < 0.001$) compared with the controls. At other concentrations of DCB of 50 µM, 100 µM and 200 µM, the protocysts production was found to be 57.6%, 36.9% and 33.4% respectively, and these effects are considered significant in comparison with Neff's or methanol as controls. As well as two sample t-test showed that the p-value (p=0.00071) for

all tested concentrations of DCB in relation to the Neff's-PG medium or 500 uM MeOH as controls.

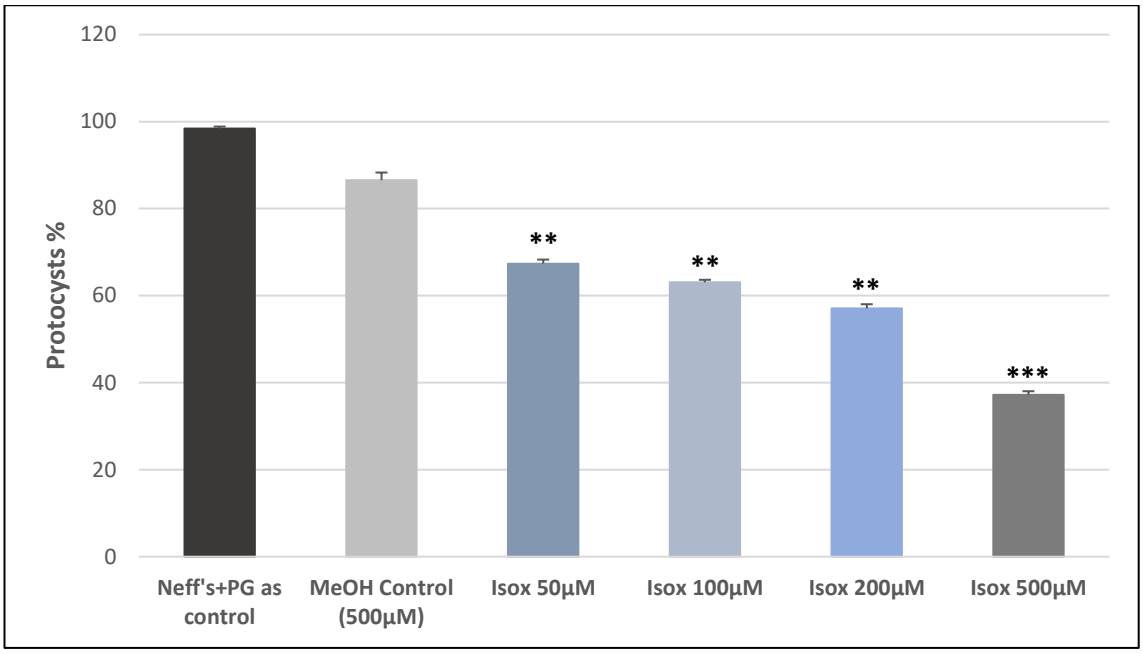

Figure 6-13: The effect of Isoxaben on the transformation of trophozoites into protocysts of *A. castellanii* (ATCC 50370) in Neff's medium plus 0.5% (v/v) propylene glycol. The haemocytometer count was performed after 24 hours of incubation. The standard error of the mean (SEM) represented the error bars (n=3). One-way analysis of variance (ANOVA) was performed. Asterisks represent values statistically significant (***P<0.001) between the tested concentrations of isoxaben and the Neff's-PG or 500 uM MeOH as controls.

The results in Figure 6.14 indicate that the effect of isoxaben on protocyst development at the higher concentration of 500 µM achieved a protocyst production level of only 37.2% and this rate was significant ($P < 0.001$) compared with the controls. Two sample t-test showed that the p-value for all tested concentrations of isoxaben compared to the controls was at p=0.0015. The production of protocysts was increased at concentrations of 50 µM, 100 µM and 200 µM, showing 67.3%, 63% and 57% respectively.

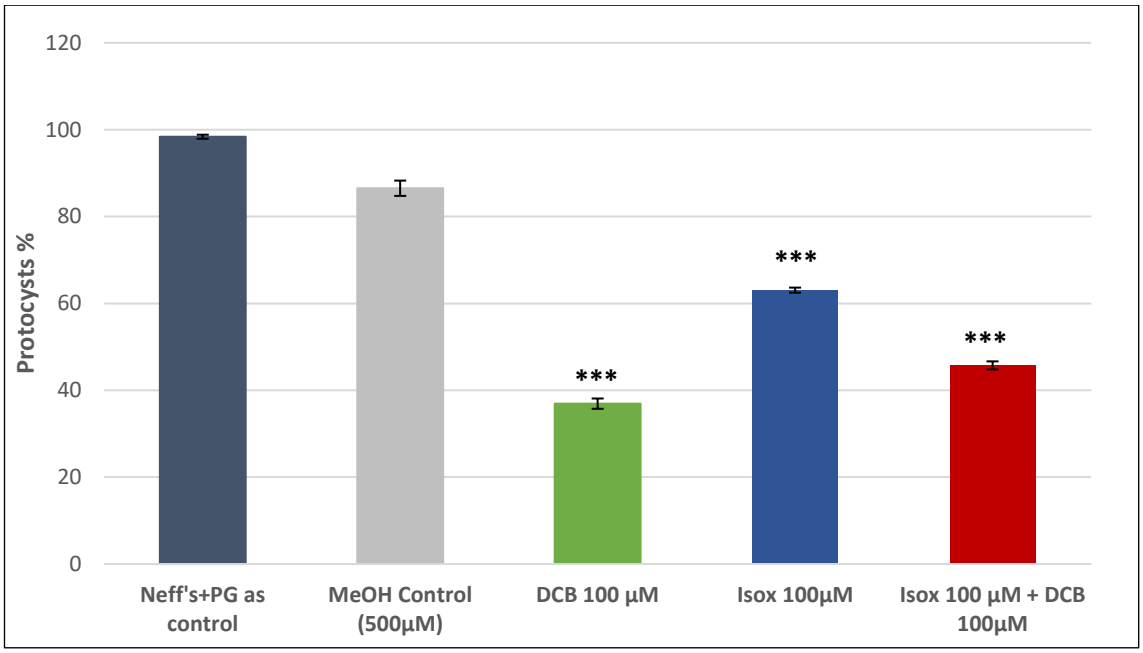

Figure 6-14: The effect of Isoxaben and DCB individual and in combination on the transformation of trophozoites into protocysts of *A. castellanii* (ATCC 50370) in Neff's medium plus 0.5% (v/v) propylene glycol. The haemocytometer count was performed after 24 hours of incubation. The standard error of the mean (SEM) represented the error bars (n=3). One-way analysis of variance (ANOVA) was performed. Asterisks represent values statistically significant (***P<0.001) between the tested concentrations of DCB and the Neff's-PG and 500 uM MeOH as controls.

The data in Figure 6.15 present the testing results for DCB and isoxaben in their own and in combination at a concentration of 100 µM on the transformation of trophozoites into protocysts. The DCB alone at 100 uM inhibited the protocysts at 36.9%. However, a significantly higher rate of protocysts production was observed at 63% for isoxaben at concentration of 100 uM. When the experiment was conducted for the combination of DCB and isoxaben at the same concentration for tested those compounds individually, a 45.7% of protocysts formation was occurred. This result could be related to an antagonistic effect in comparison to the use of DCB alone (Figure 6.15). Two sample t-test showed that the p-value=0.0025 for the tested compounds individually and in combination in comparison with Neff's-PG or 500 uM of MeOH as controls.

6.4 Discussion

This section outlines an extensive discussion about the effect of various agonists/ antagonists and cellulose synthesis inhibitors on the transformation of trophozoite into cyst and protocyst forms.

6.4.1 The encystment of *Acanthamoeba* in the presence of different agonists and antagonists

The research to date has tended to focus on the transformation of trophozoites into cysts of *Acanthamoeba in vitro* and has attempted to understand the mechanism behind the encystment process. This discussion begins with the nonspecific adrenoceptor agonists epinephrine exhibited grater encystment at 2-fold lower concentration than isoprenaline. The level of encystment that observed with selective β_1 dobutamine was close to epinephrine encystment at a 5 times lower concentration. Heaselgrave and Kilvington (2016) demonstrated that the selective β_1 dobutamine at 5-fold lower concentration to epinephrine gave 92.4%, also dobutamine gave comparable encystment to isoprenaline at 10-fold lower concentration 100µM. The potential thought for getting different results with the same concentrations of the agonists is could be related to the strain tested in the present study been affected by continuous culture or laboratory conditions. On the other hand, the specific agonist β_2 salbutamol exhibited limited agonist activity and led to encystment at a level of 36.8% and this result reflects the finding of a prior study by Heaselgrave and Kilvington (2016), who reported that this agonist has induced the encystment at a level of 50% of that observed with dobutamine, but at a 10-fold lower concentration. The effect of β_2 agonist salbutamol was probably through it utilising some activity at a β_1 receptor.

The current study demonstrated that the ultra-long agonist β indacaterol (which means this agonist has a duration of action of 24 hours) promoted the encystment in *Acanthamoeba* to level of encystment was near of that triggered by epinephrine, but the concentration was at 5-fold lower. The percentage of encystment decreased when the experiments were performed with antagonists and the largest reduction in encystment levels were found with the adrenoreceptor β_1 antagonists, metoprolol and betaxolol, giving around 30% of encystment levels. However, when the β_1 antagonists, metoprolol and betaxolol, were combined with 500 µM of epinephrine, the encystment increased up to

around 60% .These results suggest that epinephrine stimulates encystment in *Acanthamoeba*. The level of encystment was observed with a non-selective β_2 antagonist levobunolol was approximately 50% and the rate of encystment was increased slightly when this antagonist combined with epinephrine and gave around 60%. According to the results of this study, it can be concluded that the β_1 subtype is the receptor implicated in encystment of *Acanthamoeba* and these observations fit with the findings that the β_1 receptor is positively linked to the cAMP, whereas β_2 receptor is negatively linked to the cAMP.

Taken together, the results are consistent with findings of past studies by Heaselgrave and Kilvington (2016) the encystment in *Acanthamoeba castallanii* and Coppi *et al*. (2002) the encystment in *Entamoeba invadens*, which they reported that adrenoreceptor β_1 agonists and antagonists are capable of promoting or preventing the encystment process. Furthermore, in Coppi *et al*. (2002) study the authors were used a radio-labelled nonselective β adrenoreceptor agonist (^{3}H)-CGP-12177 and their findings indicated that nonlabelled adrenoreceptor antagonists, including metoprolol, might block the binding of (^{3}H)-CGP-12177 and inhibit encystment in *Entamoeba invadens*.

There is no study has examined the effect of ultra-long agonist β indacaterol and antagonists included β_1 betaxolol and β_2 levobunolol on the encystment of *Acanthamoeba*. The reason for testing betaxolol and levobunolol on the cysts formation, due these drugs used to treat glaucoma and so they are safe *in vivo* in terms of toxicity. The glaucoma's are a group of optic neuropathies defined by progressive degeneration of retinal ganglion cells which lead to visual loss (Kountouras *et al*., 2004). The contribution of the present study is obvious as the indacaterol at a 5 times lower concentration gave comparable encystment level to epinephrine. Betaxolol caused a reduction in the level of encystment of around 30% and the β_2 levobunolol had slight reduction of around 10% on the encystment rate. On the other hand, the antagonist promethazine (antihistamine) has been tested against trophozoites and cysts of *Acanthamoeba.* It was found to be amoebicidal, but its cysticidal activity occurred at higher concentrations (Baig *et al*., 2019). It has been demonstrated that bioinformatic computational and 3D modelling techniques are useful tools to determine homology for the human targets of promethazine and possibly the same targets exist for

promethazine in *Acanthamoeba* (Baig and Ahmad, 2017). Promethazine binds to the histamine H1 and dopamine D2 receptors in humans and the function of this antagonist is to block the dopamine and histamine receptors in the chemoreceptor trigger areas (Welliver, 2014).

6.4.2 The effect of magnesium chloride on the encystment of *Acanthamoeba*

The magnesium chloride ($MgCl_2$) played an essential role in mediated the encystment process of *Acanthamoeba*, as the encystment was increased a round 3-fold in comparison with the AC#6 growth medium as positive control. One of the important observation that encystment induced in the presence of Mg^{2+} ion, due this ion is capable of increase the fluidity of the lipid bilayer as a result of interacting this ion with negatively charged phospholipids leading to the alteration in the activity of membrane bound enzymes (Fuyu *et al*., 1983). Prior research has shown that the chloride ion able to be replaced for a sulphate ion without any decrease in encystment of *Acanthamoeba* (Srivastava and Shukla, 1983). It has been suggested that the divalent metal ions involving Mg^{2+} ion enhance the affinity of phosphorylase kinase enzyme (Xu *et al*., 1996), which is responsible for transforming from the inactive *b* form to the active *a* form during the encystment of *Acanthamoeba* (Heilmeyer Jr, 1991). The evidence from these findings suggested that the existence of $MgCl_2$ stimulates the encystment in *Acanthamoeba*. This process is achieved by the released of glucose from the glycogen store and producing further sugar as free for cell wall synthesis.

6.4.3 The effect of antagonists on the transformation of *Acanthamoeba* trophozoites into protocysts

The findings of the present study contribute to our knowledge by addressing the effect of different antagonists in the presence and absence of epinephrine on the conversion of trophozoites into protocysts in *Acanthamoeba*. The present investigations showed that adrenoreceptor β_1 antagonist metoprolol and β_2 antagonists including propranolol and butaxamine had a major effect on protocyst formation. Furthermore, β_1 antagonists betaxolol and β_2 levobunolol at 500 µM concentration were exhibited higher percentages of protocyst production. In addition, when these antagonists were mixed with the same concentration of epinephrine to test their effect, there was a slight increase in transformation into

protocysts in the presence of epinephrine. These results indicated that epinephrine had no effect when it combined with these antagonists and these outcomes are differ to what have seen with the cyst observations. No attempt was made to establish the potential effects of adrenoreceptor β_1 betaxolol and β_2 levobunolol. This is the first study that has demonstrate the effect of antagonists betaxolol and levobunolol on the formation of protocyst of *Acanthamoeba* and the present work is motivated by the need to block the protocyst formation of *Acanthamoeba*.

This result may be explained by the fact that the binding affinity of β_2 levobunolol for the receptor in *Acanthamoeba* is different, as this antagonist has previously shown higher affinity binging to the heart of endogenous guinea pig at 42 ± 15 nM (Sharif and Xu, 2004). *In vitro*, protocyst stage can rapidly form in the presence of propylene glycol 0.5% (v/v) and this product is a major constituent of contact lenses which impose the most risk for Acanthamoeba keratitis. The outcomes of the present study suggested that adrenoreceptor antagonists included betaxolol and levobunolol can prompt protocyst production, whereas the other antagonists included butaxamine, propranolol and metoprolol were blocked the protocyst formation. It seems that the receptor ligands in the protocyst form behave for β_2 levobunolol in the same way as observed in the encystment of *Acanthamoeba*. The current findings also support a study by Heaselgrave and Kilvington (2016), which concluded that the β_1 antagonist metoprolol and β_2 antagonists propranolol were massively inhibited encystment of *Acanthamoeba*.

6.4.4 The encystment of *Acanthamoeba* in the presence of cellulose synthesis inhibitors

In Figure 5.10, the DCB at a concentration of 500 µM blocked the encystment of *Acanthamoeba* at 17.7% compared with methanol (MeOH) at concentration of 500 µM and Neff's as a positive control which they showed encystment rate at 59.6% and 76.1% respectively. The encystment level was increased at lower concentrations of DCB at 50 µM, 100 µM and 200 µM, which gave 38.4%, 31.8% and 24.4% respectively. The results from this study showed that, at lower concentrations of 50 µM, 100 µM and 200 µM, isoxaben exhibited slight effect on the encystment process at 70.4%, 68.8% and 62.2% respectively.

Isoxaben at a concentration of 500 µM was reduced the encystment rate to 45%. Interestingly, this study demonstrated that the combination of DCB with isoxaben at a concentration of 100 µM demonstrated encystment level at 49.1% in comparison to the controls and those drugs in their own at the same tested concentration. The findings from the present study at a concentration of 500 µM from DCB in agreement with the previous research by Dudley *et al*. (2007), which reported that a top concentration of DCB at 400 µM and 480 µM produced around 19% and 15% respectively of *Acanthamoeba* encystment. When the authors tested a lower concentration of DCB at 240 µM and 320 µM, the rate of encystment found to be around 42% and 35% respectively, whereas in this study at concentration of 50 µM, 100 µM and 200 µM of DCB were proved 38.4%, 31.8% and 24.4% encystment respectively.

There are several possible explanation for getting different results: the method utilized by Dudley *et al*. (2007) contrasts to our study, those authors used a PYG medium (0.75% proteose peptone and yeast extract, glucose 1.5%, followed by the addition of 8% of glucose) to induce encystment, with 48 hours of incubation. Surprisingly in Dudley *et al*. (2007) research, 8% of glucose was added to PYG medium to promote the encystment, this observation contradicted to the findings from Byers *et al*. (1980), which reported that the encystment level of *Acanthamoeba* increased 70% up to 80% in the absence of glucose in growth media. Neff's medium was used in the present study and the incubation time was 72 hours. The strain tested in (Dudley *et al*., 2007) research was *Acanthamoeba castellanii* (ATCC 50492), while the present study tested a different strain, *Acanthamoeba castellanii* (ATCC 30868).

A study by Moon *et al*. (2015) found that, at a concentration of 400 µM, DCB demonstrated around 22% of encystment and lower concentrations of 50 µM, 100 µM and 200 µM showed around 42.9%, 44% and 39.4% of the encystment respectively. DCB has also been found to be effective in plant studies, in the past Anderson *et al*. (2002) tested DCB on two species of plants, *Lilium auratum* and *Petunia hybrida,* and found that a low concentration of DCB at 10 µmol/L caused 90% disruption of pollen tubes after only 8 hours of exposure. As can be seen in Figure 5.11, isoxaben showed minor effect on the encystment of *Acanthamoeba* at 70.4%, 68.8% and 62.2%, at concentrations of 50 µM, 100 µM and 200 µM

respectively, but the encystment of *Acanthamoeba* was decreased to 45% at a concentration of 500 µM. The findings from the present investigation agrees relatively well with that from Moon *et al*. (2015), who concluded that concentrations of 1 µM and 5 µM of isoxaben showed encystment level at around 70% and 66% respectively. When those authors increased the concentrations of isoxaben to 10 µM, 50 µM and 100 µM, the percentages of encystment were found to be around 56.6%, 61% and 64% respectively. A possible reason for the slight difference in the results between the two studies is that Moon *et al*. (2015) tested a different strain of *Acanthamoeba castellanii* (ATCC 30011) and a different incubation temperature of 25 °C, whereas the present study tested *Acanthamoeba castellanii* (ATCC 30868) and used an incubation temperature of 30 °C.

In comparison with Dudley *et al*. (2007) and Moon *et al*. (2015) studies, the current study is demonstrated for the first time that the combination of DCB and isoxaben at a concentration of 100 µM exhibited encystment at 49.1% and 45.7% for protocysts formation (as shown in figures 6.12 & 6.15). This observation could be explained by the fact that an antagonistic effect was caused when the isoxaben mixed at a concentration of 100 uM with DCB and tested on the conversion of trophozoites into cysts and protocysts. This means the ability of DCB was decreased in the combination with isoxaben compared to the DCB effect alone on the encystment rate and protocysts formation. it seems possible that this result is due to DCB blocking the cellulose synthase enzyme and preventing the cyst and protocyst formation. Isoxaben and DCB both target cellulose synthase enzyme which is involved in the cellulose biosynthesis during the encystment and protocysts production in *Acanthamoeba*. Based on the findings obtained from this study that the DCB particularly the 500 uM concentration was inhibited the transformation of trophozoites into cysts at 17.7% compared with the same tested concentration 500 uM of isoxaben which showed only 45% encystment. There are three different explanations for the poor activity of isoxaben on *Acanthamoeba* included: firstly, it could be due to the isoxaben not being transported to the active site of the cellulose synthase enzyme for *Acanthamoeba*. Second, the isoxaben may not be taken up by *Acanthamoeba*. Third, the isoxaben might not be inhibiting the active sites of cellulose synthase enzyme during the transformation of trophozoites into cysts or protocysts.

In the plants, Isoxaben targets certain CesA proteins and leads to a reduction in this protein in the cellular membrane (Heim *et al*., 1991, Peng *et al*., 2002). The effect of isoxaben in plants is most likely that it binds to the amino acid included glycine and threonine which aid the process of cellulose synthesis (Desprez *et al*., 2002, Scheible *et al*., 2001). On the other hand, the effect of DCB appears to be to disrupt the cellulose synthase complex subunits (CSCs) in the plasma membrane and is also involved in blocking the CSCs in the process of cellulose synthesis (Doblin *et al*., 2002, Mutwil *et al*., 2008). The mechanism of action of DCB in plants is that it causes alterations in cytoskeleton organisation in *Arabidopsis* thaliana (Peng *et al*., 2001). Possibly the mechanism for DCB in *Acanthamoeba* is similar to that working in plants by distribution of the cytoskeleton of *Acanthamoeba* or the inhibition of glucose polymerization, preventing the formation of cellulose in the cyst wall during the encystment process. The main elements of the cytoskeleton of *Acanthamoeba* are the fibres and networks of actin that are organized in cytoplasmic locomotion (González-Robles *et al*., 2008). Furthermore, It has been reported by Dudley *et al.* (2009) that the encystment of *Acanthamoeba* could be blocked by using particular inhibitors, such as cytochalasin D and Y27632 and the findings from their investigations indicated that cytoskeleton re-arrangements play a crucial role in the encystment process.

6.4.5 The effect of cellulose synthesis inhibitors on the transformation of trophozoites into protocysts

Isoxaben and DCB were tested in this study to assess their effect on the protocyst formation. The results in Figure 6.13 suggest that the correlation between higher and lower concentrations of DCB is interesting as the production of protocysts was further reduced at a concentration 500 µM which produced 24.6%. The effect of DCB on the protocysts formation was slightly decreased at concentrations of 50, 100, 200 µM, giving 57.6%, 36.9% and 33.4% protocysts production respectively.

The present study has demonstrated for the first time that the effects of DCB on protocysts formation in *Acanthamoeba* and the results are in line with prior study conducted by (Moon *et al*., 2015), who found that a concentration of DCB at 50 µM, 100 µM, 200 µM and 400 µM gave around 42.9%, 44%, 39.4% and 22%

respectively of *Acanthamoeba* encystment. The novel observation of the current study is that the combination of isoxaben with DCB at a concentration of 100 µM gave protocysts formation at 45.7% compared with isoxaben and DCB as individual which produced protocysts level at 63% and 36.9%. The combination results refer to antagonistic effect related to the activity of DCB alone. Fugelstad *et al*. (2009) found that, at a concentration of 200 µM, DCB inhibited the growth of *Saprolegnia monoica* mycelium. After 4 days, DCB inhibited around 40 and 60 % of growth compared with the positive controls of methanol and water, respectively. It seems possible that these results are due to the blocking effect of DCB on cellulose synthases complex in moulds which has an impact on *Saprolegnia monoica* growth.

It can be seen from the data in Figure 6.14 that isoxaben at concentrations of 50 µM, 100 µM and 200 µM inhibited the development of protocysts by 67.3%, 63% and 57% respectively. These results are in the line of Moon *et al.* (2015) research that found at concentrations of 10, 50 and 100 µM of isoxaben exhibited encystment rate at around 56.6%, 61% and 64% respectively. Furthermore, the protocyst formation decreased at the higher concentration of 500 µM of isoxaben, giving a level of protocyst of 37.2%. The results from this study indicate that DCB inhibited the conversion of *Acanthamoeba* trophozoites into protocysts, on the basis of this data, it is possible to hypothesise that the mechanism of action for DCB in *Acanthamoeba* protocyst is similar to that operating in cysts by inhibiting the cellulose synthase enzyme. The most surprising findings of this study relate to the isoxaben results, as this herbicide showed only slight inhibition of *Acanthamoeba* encystment even at a higher concentration of 500 µM compared with DCB. When isoxaben was combined with DCB at a concentration of 100 µM, the formation of protocysts was found to be 45.7% compared with the use of DCB which at the similar tested concentration it gave 36.9%. This suggested a potential antagonism impact on the use of two compounds compared with tested of DCB alone. The resistance of *Acanthamoeba* to isoxaben during the transformation of trophozoites into protocysts might be related to the same reasons which I clarified in the sub-heading 6.4.4 above.

6.5 Future research work

Future studies should be carried out to isolate and sequence the receptor which the β ultra- long agonist, β_1 adrenoreceptor agonists and antagonists interact with and investigate how this receptor is involved in the encystment process of *Acanthamoeba*. Further experimental investigation is needed to assess the effects of irreversible antagonists such as pindobind on the transformation of trophozoites into cysts, as this antagonist can irreversibly bind to the receptor for a long period and it will not be able to respond to endogenous catecholamine of *Acanthamoeba*. Conversely, when the antagonist combined with epinephrine it competed for the receptor and had an antagonistic effect and the reason for that is due epinephrine is in a dynamic equilibrium and it associated and disassociated from the receptor in *Acanthamoeba*. The findings from this investigation established that a micromolar concentration of the adrenoreceptor β ultra-long agonist indacaterol promoted the encystment process in *Acanthamoeba*. Considerably more work will need to be done to isolate the receptor from the trophozoite membrane lysate by using indacaterol as a ligand for the receptor along with a bead-based column approach. It would be interesting to perform N-terminal sequencing of the receptor/protein structure using the SDS page and further analysis could also be undertaken to characterise the receptor/protein using LC/MS protein analysis. The outcomes of the recently sequenced *Acanthamoeba* genome confirmed that no sequence shares homology with well-known mammalian adrenergic receptor systems and a research conducted by Clarke *et al*. (2013) reported that the *Acanthamoeba* genome encoded 35 G-protein-coupled receptors are involved in cell signalling.

6.6 Conclusion

The results of the present study demonstrate that the agonists and antagonists promoted and blocked both the encystment process and the transformation of trophozoites into protocysts. This is the first study to investigate the effect of a number of adrenoceptor agonists and antagonists on the conversion of trophozoites into protocysts and this research has gone some way towards enhancing our understanding of how these agonists and antagonists work in *Acanthamoeba*. Overall, the results of the current study indicate that the β_1 and α/β agonists, including dobutamine, indacaterol and epinephrine, promote

encystment in *Acanthamoeba*, whereas β_1 and β_2 antagonists, including metoprolol, propranolol and betaxolol, inhibit the encystment process. The findings from the protocyst investigations reveal that the β_1 and β_2 antagonists, including metoprolol, propranolol, and also butaxamine, prevent the formation of *Acanthamoeba* protocysts, while the β_1 levobunolol and betaxolol stimulate protocysts production. Targeting cellulose biosynthesis in *Acanthamoeba* is very important, due to the failure of the current drug regimen to kill the cysts of *Acanthamoeba* and recurrent the infection. The findings from this study showed that DCB blocked both the encystment and protocysts production. The mechanism of action for DCB in *Acanthamoeba* is blocking the cellulose synthase enzyme which is involving in catalysing cellulose biosynthesis pathway. This study offers the potential of using DCB as an effective treatment against both cysts and protocysts production of *Acanthamoeba in vitro*. However, DCB and isoxaben compounds are soluble in methanol and may be not safe to use for the treatment of AK *in vivo*. A minor inhibition was observed from isoxaben on the encystment and protocysts formation, but the combination of isoxaben and DCB at a concentration of 100 µM reduced the encystment and protocysts formation. However, the combination results could be referred to antagonistic effect compared with the use of DCB alone on the conversion of trophozoites into cysts and protocysts.

Chapter Seven

Examining the carbohydrates composition of cyst and protocyst walls of *Acanthamoeba*

Chapter 7: Examining the carbohydrates composition of cyst and protocyst walls of *Acanthamoeba*

7.1 Introduction

As detailed previously in chapter 1, section 1.3.2, under unfavourable conditions such as lack of food, hyperosmolarity, the trophozoites of *Acanthamoeba* switch to another form which is the cyst with a thick wall and makes the organism highly resistant to the disinfection and able to survive in adverse environmental conditions for many years.

7.1.1 Composition of the walls of *Acanthamoeba* cysts and protocysts

At 35%, carbohydrates form the highest percentage of the cyst wall structure of *Acanthamoeba*, proteins form 33%, lipids 4-6%, 8% ashes (dust) and 20% is composed of undetermined materials (Neff and Neff, 1969). Data from past studies suggested that the exocyst of *Acanthamoeba* mainly comprises hydroxyproline-rich, acid-resistant proteins but cellulose and lipids were also detected in this layer (Barrett and Alexander, 1977, Bowers and Korn, 1969). Cellulose is mainly found in *Acanthamoeba* endocysts and earlier literature demonstrated that cellulose represents around 10% of the total dry weight of the cyst wall (Tomlinson and Jones, 1962). However, a more recent study (Garajová *et al*., 2019) has reported that the cellulose fibrils exist in both layers the endocyst and ectocyst of the *Acanthamoeba* cyst wall.

The sugar composition of *Acanthamoeba* cyst walls has been investigated using fluorescein labeled lectins and it has been found that glucose, mannose, galactosamine and glucosamine residues are present (Elloway *et al*., 2004, Magistrado-Coxen *et al*., 2019). In another study utilising gas chromatography, coupled with a mass spectrometry (GC-MS) instrument, Dudley *et al*. (2009) showed it was possible to analyse the sugar moieties and glycosidic linkages in the cyst walls and trophozoites of *Acanthamoeba castellanii* (ATCC 50492) which belongs to the T4 genotype and the carbohydrates were found to form 0.7% of the components of cyst walls. The significant difference between the amounts of carbohydrates (35%) reported above and the chemically treated cysts (0.7%) is

most likely caused by the loss of intracellular contents such as glycogen (Dudley *et al*., 2009). The chemical analysis of glycosidic linkages for cyst and trophozoite is important in terms of pharmaceutical therapeutics approach to design a new agents for AK infection.

GC-MS analysis of the glycosidic linkages of *A. castellanii* (ATCC 50492) cyst walls showed that there are 1,4-linked glucosylpyranose representing (22%) which is an obvious sign of cellulose, and the percentage increased to (28.6%) for 1,3-linked galactopyranose (galactose linkage polymer). Other linkages were identified, including 3,4-linked galactopyranose (13.6%) (galactose linkage polymer), 4,6-linked mannopyranose (7.8%) (mannose linkage polymer), 3,6-linked galactopyranose (7.2%) (galactose linkage polymer), 5-linked xylofuranose (7%) (xylose linkage polymer), 3,4-linked glucopyranose (6%) glucose linkage polymer), 2,4-linked gluco or galactopyranose (4.4%) (glucose/galactose linkage polymer) and terminal mannopyranose (3.2%) (Dudley *et al*., 2009).

Under the laboratory conditions the trophozoite of *Acanthamoeba* is transformed into protocyst form by incubating the trophozoite in Neff's medium plus 0.5% (v/v) propylene glycol in an orbital shaking incubator at 32°C and 120 rpm for 2 hours. The propylene glycol (PG) contributes in the transformation of *Acanthamoeba* trophozoite into a protocyst form. When the PG is added to the Neff's medium it has a significant role in the transformation process; it may interfere with the plasm membrane of *Acanthamoeba* and resulted in protocyst stage of this organism. There has been no detailed investigation of the structure of protocyst walls. However, a study conducted by Kliescikova *et al*. (2011) used various lectins to detect the presence of specific sugars within cyst and protocyst walls of *Acanthamoeba*. The authors showed that the protocyst cell wall reacted only with Concanavalin A lectin, which suggests that only α-glucose and mannose are present, whereas the exocyst layer of the cyst walls was reacted with all lectins which suggested that the ectocyst layer of cyst walls mainly consists of protein. The protocyst is unlike the mature cyst, it's cell wall appears under transmission electron microscopy as a single layer (Kliescikova *et al*., 2011).

7.1.2 The presence and structure of cellulose and chitin polymers

Cellulose is the most plentiful natural polymer on earth, a particular product of solar energy because of its source in the photosynthetic process (Klemm *et al*., 2005). Cellulose is the most important component of plant biomasses. It is also generated in significant amounts by green algae, such as *Micrasterias*, a Gram-negative soil bacterium *Acetobacter xylinum*, slime mold *Dictyostelium*, sea animals such as *Halocynthia* and protists, such as the cyst walls of *Acanthamoeba* (Schwarz, 2001, Lynd *et al*., 2002, Bishop *et al*., 2002). Around 30 distinct cellulose molecules join together and produce major units referred to as 'primary fibrils'. These fibrils assemble into larger units known as 'microfibrils' and hydrogen bonds hold together the chains of cellulose microfibrils (Brown Jr and Saxena, 2000, Lynd *et al*., 2002).

The chemical hydrolysis of cellulose polymer into glucose monomers is cheaper than the digestion by enzymes and requires acids, such as hydrochloric acid (HCl) and sulfuric acid (H_2SO_4). It has been reported that H_2SO_4, at a concentrations of 1.0 M & 2.5 M, gives 12% & 24% yield respectively of glucose after treatment for 2 hours at 100 °C. Hydrolysis with HCl under the same conditions as those used for H_2SO_4, gives lower percentage yields of glucose, at 10 and 19% respectively (Hutomo *et al*., 2015). A possible explanation for this difference H_2SO_4 is a stronger than HCl. The chemical structure of the cellulose polymer is made up of D-glucose monomers, which are compressed through β-1,4 glycosidic bonds (Figure 7.1). β D-glucose is an essential subunit of the cellulose polymer and is the key element of the cyst wall in *Acanthamoeba*.

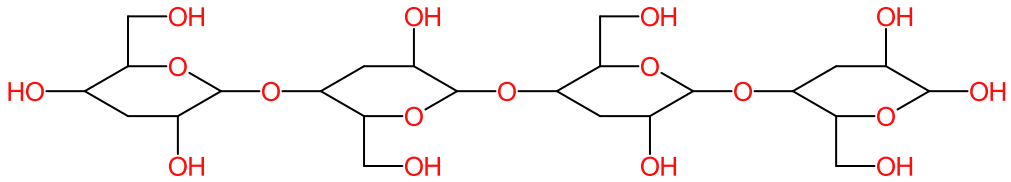

Figure 7-1: The chemical structure of cellulose polymer, which is made up of repeating glucose monomers.

The rationale for providing a review for chitin is because we hypothesised that the *Acanthamoeba* protocyst consists of *N*-acetylglucosamine. Chitin is a polymer comprising *N*-acetylglucosamine linked by covalent β-1,4 glycosidic bonds and is present in the cell walls of plants and fungi, in the exoskeletons of insects such as Arthropoda such as mosquitos, in the cyst walls of some protozoa, such as the species *Giardia intestinalis* and *Entamoeba histolytica* and on the cell surface of *Trichomonas vaginalis, Phytomonas nordicus* and *Blastocystis hominis* (Martínez *et al*., 2001). Earlier studies have found a high amount of chitin, at around 25%, in the cyst wall of *Entamoeba invadens* and also the chitin synthase enzyme is expressed in this organism during the encystment process (Arroyo-Begovich *et al*., 1980, Arroyo-Begovich and Cárabez-Trejo, 1982). The chemical structure of the chitin polymer consists of repeating units of *N*-acetyl-glucosamine linked by covalent *β*-1,4 glycosidic bonds and it has free amido groups, as shown in Figure 7.2.

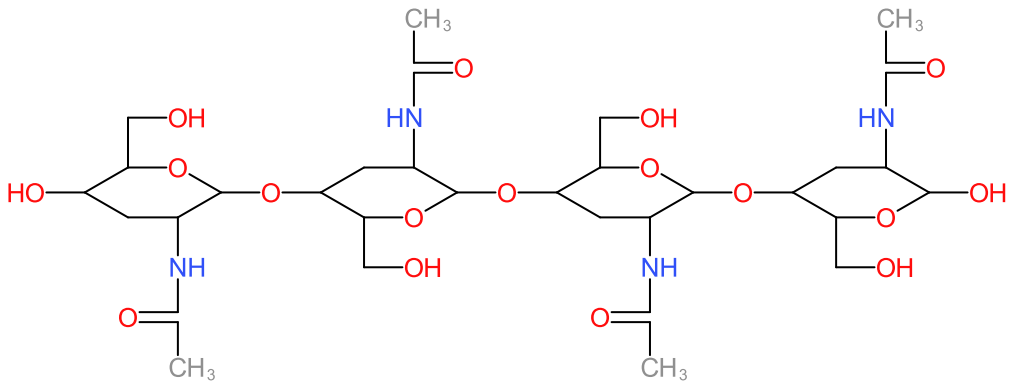

Figure 7-2: Chemical structure of chitin polymer, which comprises repeating *N*-acetylglucosamine monomers.

7.1.3 Aim and objectives of this chapter

The aim of this chapter is to investigate and confirm the potential existence of a 3rd life cycle stage of *Acanthamoeba*, by differentiating the sugar composition of protocyst wall in *Acanthamoeba castellanii* (ATCC 50370) and the cyst wall using LC/MS analysis. To achieve this aim, the following objectives were set:

V. Prepare solutions of standard sugars for LC/MS analysis for comparison purposes.
VI. Undertake acid hydrolysis and LC/MS analysis for cellulose/chitin as standards to compare with cyst and protocyst samples.
VII. Perform enzymatic treatments for cysts and protocysts to digest the intercellular components.
VIII. Conduct acid hydrolysis and LC/MS analysis for cyst and protocyst walls to determine the sugar types.

7.2 Materials and Methods

7.2.1 Analysis of sugar standards by LC/MS

These experiments were performed on standard sugars to ensure that the instrument worked and to validate the method used to separate the sugars. All the standard sugars were obtained from VWR (Lutterworth, Ltd, U.K.) and the purity for all sugars is ≈98%. The samples were prepared and analysed by adapting the procedure used by Bawazeer *et al*. (2017). The mobile phase was prepared by weighing out 10 mg of the standard sugars and the powder was dissolved in 10 mL of acetonitrile (ACN) (LC-MS grade) and water (50:50) (Sigma, Dorset, U.K.). The samples were placed in LC/MS glass vials and transferred to the LC-MS instrument (Agilent 6200 Series TOF/6500 Series Q-TOF system) and the analysis was carried out by Surila Darbar, a technician in the Chemistry and Analytical Service of the Department of Biology, Chemistry and Forensic Science at the University of Wolverhampton. The separation was achieved on a ZIC®-HILIC column (150 x 4.6 mm, 3.5 µm particle size, supplied by Sigma, Dorset, U.K.), the injection volume was 0.5 µL and the flow rate was 0.1 mL/min.

7.2.2 Hydrolysis of cellulose and chitin

To prove that the hydrolysis procedure worked, the cellulose and chitin polymers were obtained from (Sigma, Dorset, U.K.) and hydrolysed using a method modified from that proposed by Morales-delaRosa *et al*. (2014). Initially, 250 mg of cellulose or chitin were added to a sealed tube containing 20 mL of distilled water and the analyte was vortexed. The mixture was transferred to a tight steel container reactor and heated to 140 °C in an oven incubator for 1 hour. Subsequently, 5 mL of distilled water containing 0.2 M and 1.1 M concentrations of H_2SO_4 were added in drops to the chitin and cellulose respectively to give the final concentration of 1.1 M for cellulose and 0.2 M for chitin, and then the reaction time of the samples was recorded from this point for 2 hours. After 2 hours, the mixture of the samples was immediately cooled with ice -20°C in order to stop the reaction and the samples were centrifuged at 3000 × *g* for 10 min. The hydrolysed samples were filtered using a vacuum filter (0.2 µm) to remove the insoluble materials.

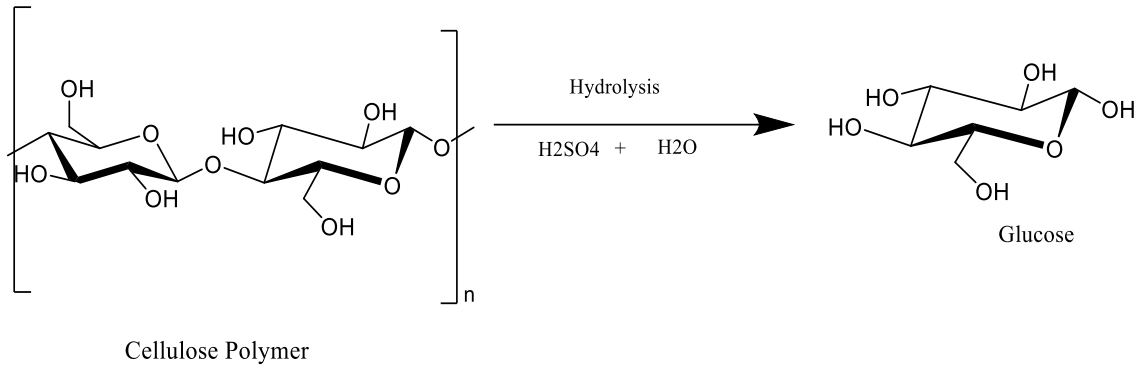

Figure 7-3: Hydrolysis of cellulose polymer in H_2SO_4 and distilled water at a concentration of 1.1 M into glucose monomers.

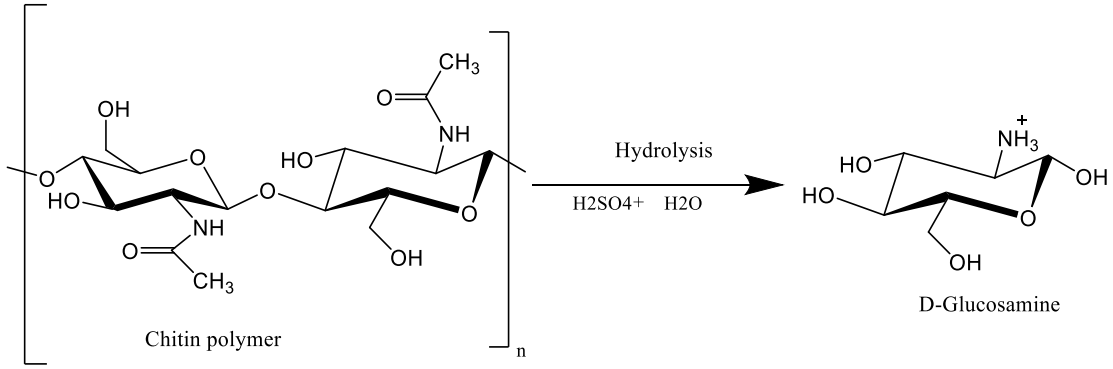

Figure 7-4: Hydrolysis of chitin polymer in H_2SO_4 and distilled water at a concentration of 0.2 M into monomers of D-glucosamine.

7.2.3 LC-MS analysis of the hydrolysed samples of cellulose and chitin

The mobile phase was prepared by diluting the hydrolysed cellulose and chitin samples in acetonitrile and water (50:50) to a concentration of 1 mg/mL. As detailed in section 7.2.1 above, the separation was accomplished on a ZIC®-HILIC column (150 x 4.6 mm, 3.5 µm particle size, Sigma, Dorset, U.K.). The injection volume was 0.5 µL and the flow rate was 0.1 mL/min.

7.2.4 Enzyme treatments of the cysts and protocysts

Cysts and protocysts were prepared as detailed in chapter 2, sections 2.8 – 2.9. The experiments followed the method previously described by Gerwig *et al*. (2002) and Jarroll *et al*. (1989) in relation to *Giardia* with modifications. Around 1 x 10^7 cells/mL of cysts or protocysts of *Acanthamoeba castellanii* (ATCC 50370) were washed in a ¼ strength Ringer's solution and centrifuged at 1000 x *g* for 5

min. The pellets were suspended in 10% sodium dodecyl sulphate (SDS) (to solubilize the intracellular components) and heated up in a 100 °C water bath for 5 min and then washed in distilled water until there were no visible indications of detergent remaining. This step was repeated once, and the supernatant was discarded. The resulting cyst or protocyst pellets were resuspended in amyloglucosidase buffer (20 mM acetate buffer, pH 4.5), and transferred to a centrifuge tube. Subsequently, 80 units of amyloglucosidase (13.3 mg) were added to the centrifuge tube on a rotary shaker in a 55 °C warm incubator and the samples were stirred for 60 min (to break down the linkages between saccharides). This step was repeated once, and the supernatant was discarded. The cyst or protocyst pellets that resulted from the amyloglucosidase treatment were washed in papain buffer (100 mM phosphate buffered saline (PBS), pH 7.2, with 5 mM cysteine and 5 mM ethylenediaminetetraacetic acid (EDTA) and then the pellets were resuspended in 3 mL of papain buffer and again placed in a centrifuge tube. The samples were stirred with 50 units of papain (157 µg) for 2 hours at 60 °C (to digest the intracellular elements). This step was repeated again for one time, and the supernatant was discarded.

The next step was to collect the cyst or protocyst pellets by centrifuging at 21,000 × *g* for 30 min and the samples were then washed with 10% SDS and distilled water. After the papain treatments, the samples were subjected to DNase and RNase treatments by resuspending the pellets in 3 mL of XNase buffer (0.1 M PBS, pH 7.2, with 5 mM $MgCl_2$) in a centrifuge tube. The samples were incubated with 500 kunitz units of DNase (1.25 mg) and 80 kunitz units of RNase (1.6 mg) (to degrade the DNA and RNA) and stirred for 2 hours at 37 °C. These treatments were repeated once, and the pellets were washed with amyloglucosidase buffer. The next step was the centrifugation of the pellets at 21,000 × *g* for 30 min at 10 °C (in order to separate the cell walls from the other components). After the DNase/RNase treatments, the pellets subjected to further amyloglucosidase treatments as described above. The pellets were washed with proteinase K buffer (50 mM Tris, pH 8.0, 0.2% Triton X-100, 1 mM $CaCl_2$). The resulting pellets were suspended in fresh proteinase K buffer, 50 units of proteinase K (1.6 mg) (to degrade the protein) were added and the samples were stirred for 2 hours at 60 °C. This step was repeated, and the supernatant was discarded. Then the pellets were washed with an equal volume of 10% SDS containing 100 mM of

dithiothreitol for 5 min at 100 °C (to remove the intercellular components). After the enzymatic treatments, the resulting pellets were washed five times in distilled water to eradicate the detergent and dithiothreitol. The final step of the procedure was lyophilizing the resulting pellets in a freeze dryer (to remove the distilled water) for 48 hours and then the dry powder was subjected to the process of hydrolysis.

7.2.5 Hydrolysis and LC-MS analysis of the cyst and protocyst walls

As detailed in sections 7.2.2 and 7.2.3 describing the hydrolysis and LC-MS analysis of cellulose/chitin, the same method of hydrolysis and LC-MS conditions were employed for the cysts and protocysts with some modifications. First, 20 mg of enzyme treated lyophilised cyst or protocyst were added to tubes then sealed containing 3 mL of distilled water and the analyte was vortexed. The suspension was heated to 140 °C in an oven for 1 hour and then 2 mL, containing 0.2 M and 1.1 M concentrations of aqueous H_2SO_4, were added in drops to the protocysts and cysts respectively to give the final concentration of 1.1 M for cyst and 0.2 M for protocyst and the samples were heated to 140 °C for 2 hours. After 2 hours of hydrolysis, the reaction was stopped, and the samples were cooled in ice -20°C. When it was observed by a visual that the hydrolysed solution of cyst and protocyst samples was completely soluble, the solution was centrifuged at 3000 × *g* for 10 min and then filtered using a syringe filter (0.2 µm). The mobile phase was made up by diluting the hydrolysed solution of cysts and protocysts with acetonitrile and water (50:50, LC/MS grade) to a concentration of 1 mg/mL and the separation was performed under the same conditions as described in sections 7.2.1 and 7.2.3 above.

7.3 Results

This section presents the results from the inverted light microscopy images showing the morphology changes of cysts and protocysts after enzyme treatments, LC-MS data for sugar standards analysis, hydrolysed cellulose/chitin as standards, and the sugars type in the hydrolysed cyst and protocyst walls.

7.3.1 Observations from inverted light microscopy

Significant changes occurred in the morphology of NNA cysts and protocysts of *Acanthamoeba castellanii* (ATCC 50370) after enzymes treatment, as shown in Figure 7.5. Healthy cysts with a clear double cell walls can be observed in Figure 7.5.A. Figure 7.5.B. shows the cysts after being treated with different enzymes and the intercellular constituents were removed. Healthy protocysts with single layers can be seen in Figure 7.5.C. However, when the protocysts were exposed to the enzyme treatments, all the intercellular elements were eradicated and only a single layer of protocyst remained, as shown in Figure 7.5.D.

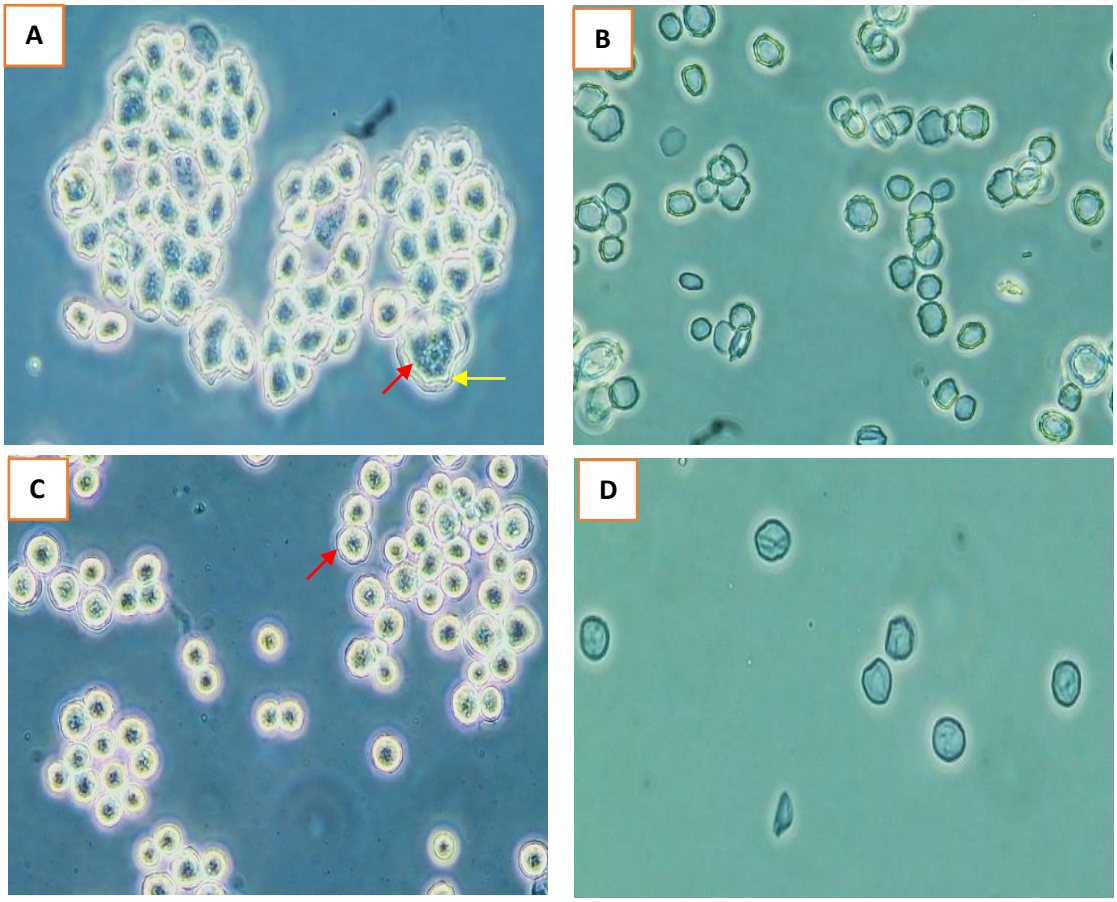

Figure 7-5: Inverted light microscopy images of *Acanthamoeba castellanii* (ATCC 50370). (A) Healthy NNA cysts as control with double cell walls the endocyst (red arrow) and ectocyst (yellow arrow) (B) Treated NNA cysts with several enzymes (C) Untreated protocysts as control with single cell wall (red arrow); (D) Enzymes treated protocysts. The magnification for all images is ×400.

7.3.2 LC-MS analysis of standard sugars

Preliminary experiments were performed to confirm that the column adequately separated the sugar components. For these experiments, a ZIC®-HILIC HPLC column was utilised, and this column contains a zwitterionic stationary phase covalently attached to porous silica. The reason for used this particular column, due it has hydrophilic zwitterion makes the column suitable for hydrophilic compounds (carbohydrates) and enhances the peak resolution of the analyte. The standard sugars are isomers, which they have an identical molecular formula (the same number of atoms of each component), but the structure is different. The blank can be observed in Figure 7.6.B. As illustrated in Figures 7.6.C & D, the molecular ion peak for glucose as a standard was found to be 203.056 m/z and it was detected within a retention time of 3.675 min. The other two sugars, mannose and galactose, showed comparable molecular ion peaks of 203.054 m/z and 203.057 m/z, with retention times of 3.658 min and 3.682 min respectively, as presented in Figures 7.6.E and F & G and H.

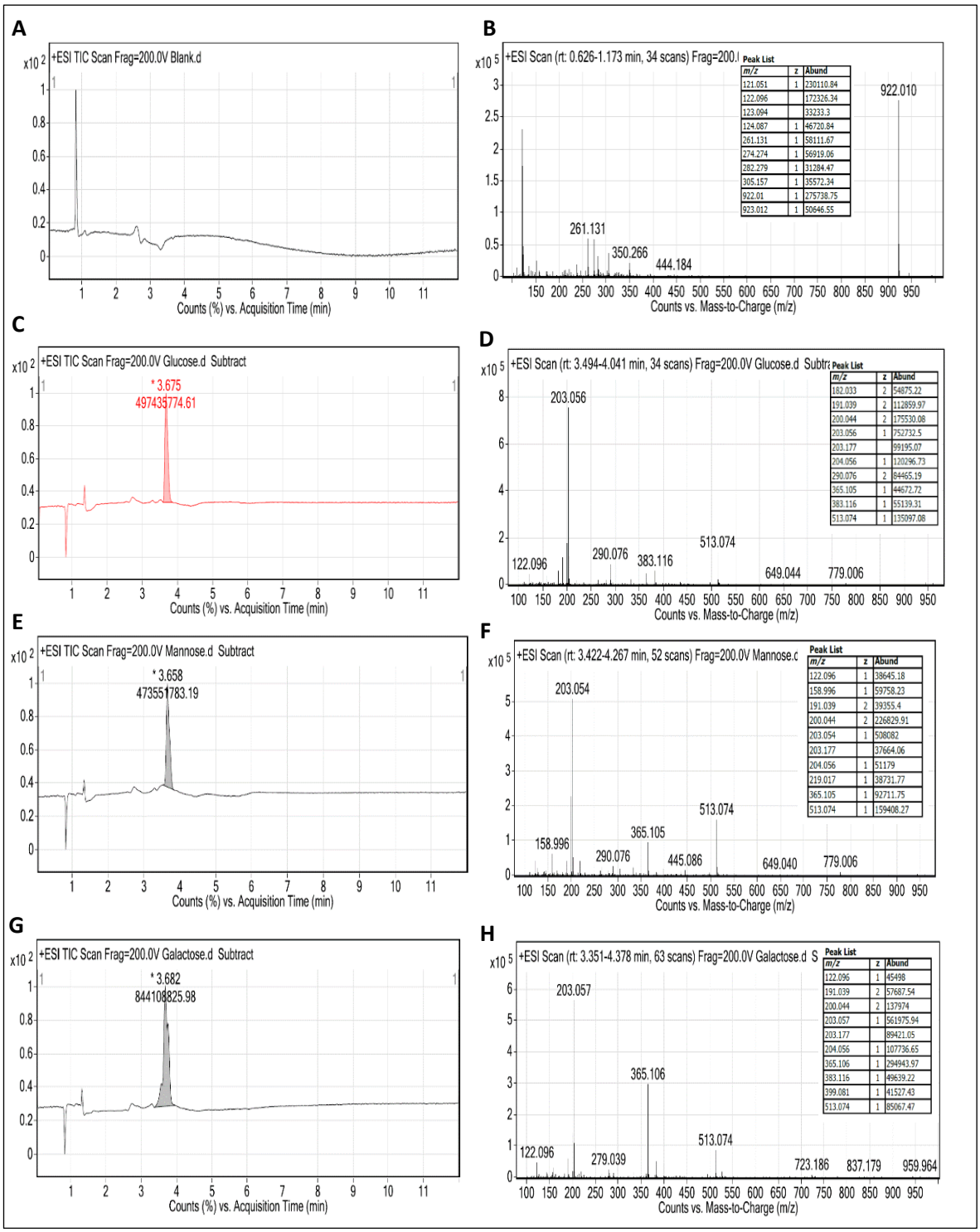

Figure 7-6: Separation of standard sugars: (A) Retention time for blank sample, (B) Extracted ion chromatogram peak for blank sample, (C) Retention time for glucose, (D) Separated ion chromatogram peak for glucose, (E) Retention time for mannose, (F) Extracted ion chromatogram peak for mannose, (G) Retention time for galactose, (H) Obtained ion chromatogram peak for galactose.

The same molecular ion peaks were obtained from LC/MS analysis for ribose and xylose, at 173.042 m/z, with different retention times of 3.666 min for xylose (Figure 7.7.B) and 3.658 min for ribose (Figure 7.7.D). *myo*-Inositol was run as a control and, with a retention time of 3.831 min, it gave a molecular ion peak at 203.059 m/z. *N*-acetyl-*D*-glucosamine was detected to have a molecular ion peak of 244.086 m/z and a retention time of 3.460 min (Figure 7.7.G & H). A number of peaks have been observed in the MS spectra, and those peaks are known to be background instrumental noise; each individual sugar can only give one molecular ion peak.

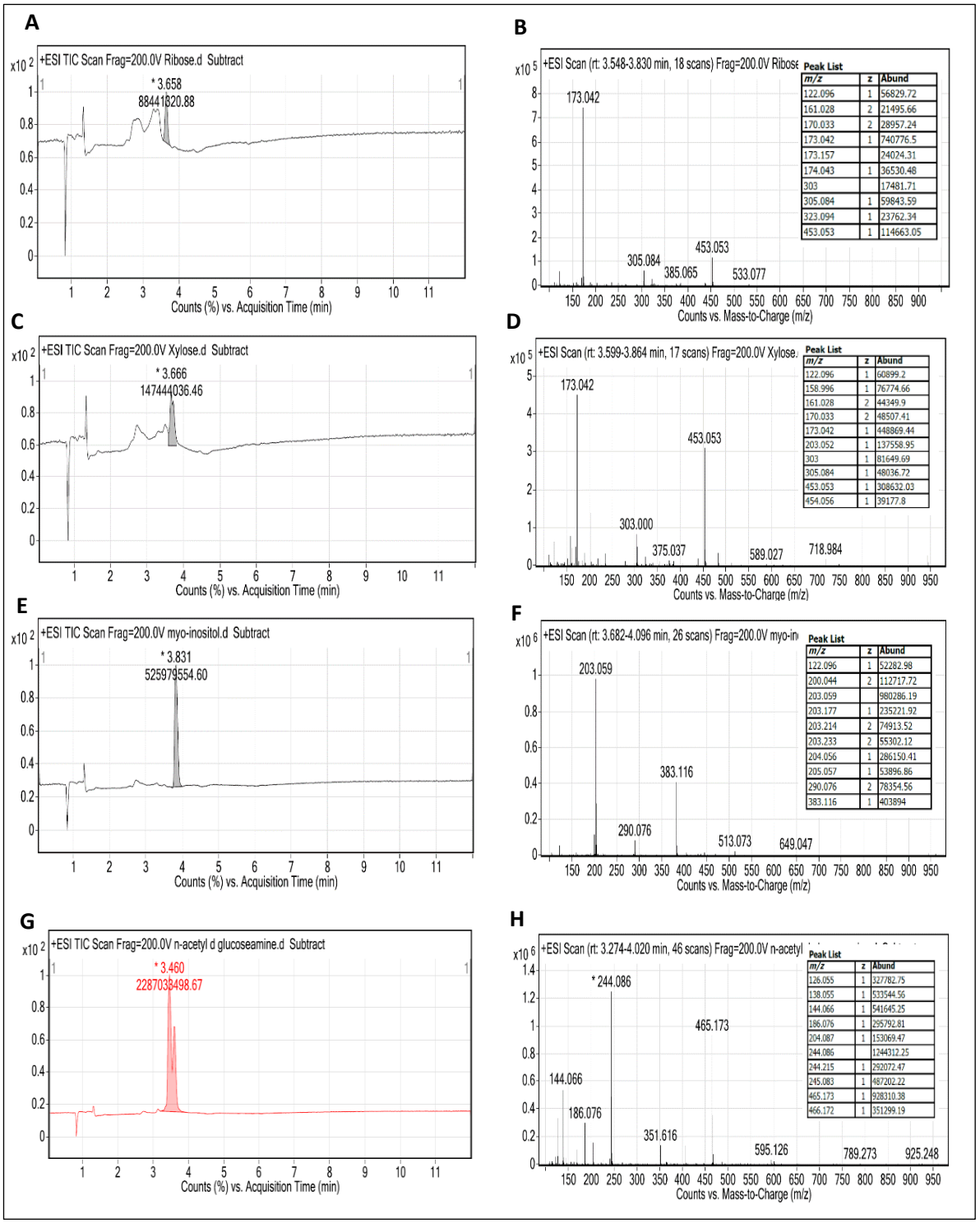

Figure 7-7:Separation of standard sugars: (A) Retention time for ribose, (B) Extracted ion chromatogram peak for ribose, (C) Retention time for xylose, (D) Obtained ion chromatogram peak for xylose, (E) Retention time for myo-inositol, (F) Extracted ion chromatogram peak for myo-inositol, (G) Retention time for N acetyl-D-glucosamine, (H) Separated ion chromatogram peak for *N*-acetyl-d glucosamine.

7.3.3 LC/MS analysis of cyst and protocyst walls

The retention time for the glucose which was extracted from the hydrolysed cyst wall in 1.1 M of aqueous H_2SO_4 was 3.402 min and the molecular ion peak was found to be 203.020 m/z as shown in Figure 7.6. A & B. This is consistent with the expected result, glucose from the cellulose of the cyst cell wall of *Acanthamoeba*. Interestingly, in liquid chromatography (LC) analysis of the hydrolysed protocyst wall sample (prepared in 0.2 M of aqueous H_2SO_4) peaks related to two different components were observed. The first component was detected with a retention time of 3.712 min and the mass spectrometry (MS) ion peak for this fragment was observed at 203.023 m/z (Figure 7.6. C & D) which is consistent with the ion peak of the glucose used as a standard in terms of molecular ion, but the retention time is slightly different as the standard of glucose was detected within a retention time of 3.675 min. The second component from the hydrolysed protocyst wall in in 0.2 M of aqueous H_2SO_4 was identified from LC with different retention time of 4.139 min, and molecular ion of 236.937 m/z (Figure 7.6.E & F). Based upon the mass data, the structure for this fragment ion may be in line with a methylated analogue of *N*-acetylglucosamine; more analysis is required in the future to unequivocally characterize this component. The presence of these two different components clearly indicates a difference between the protocyst and the cyst of *Acanthamoeba castellanii* (ATCC 50370).

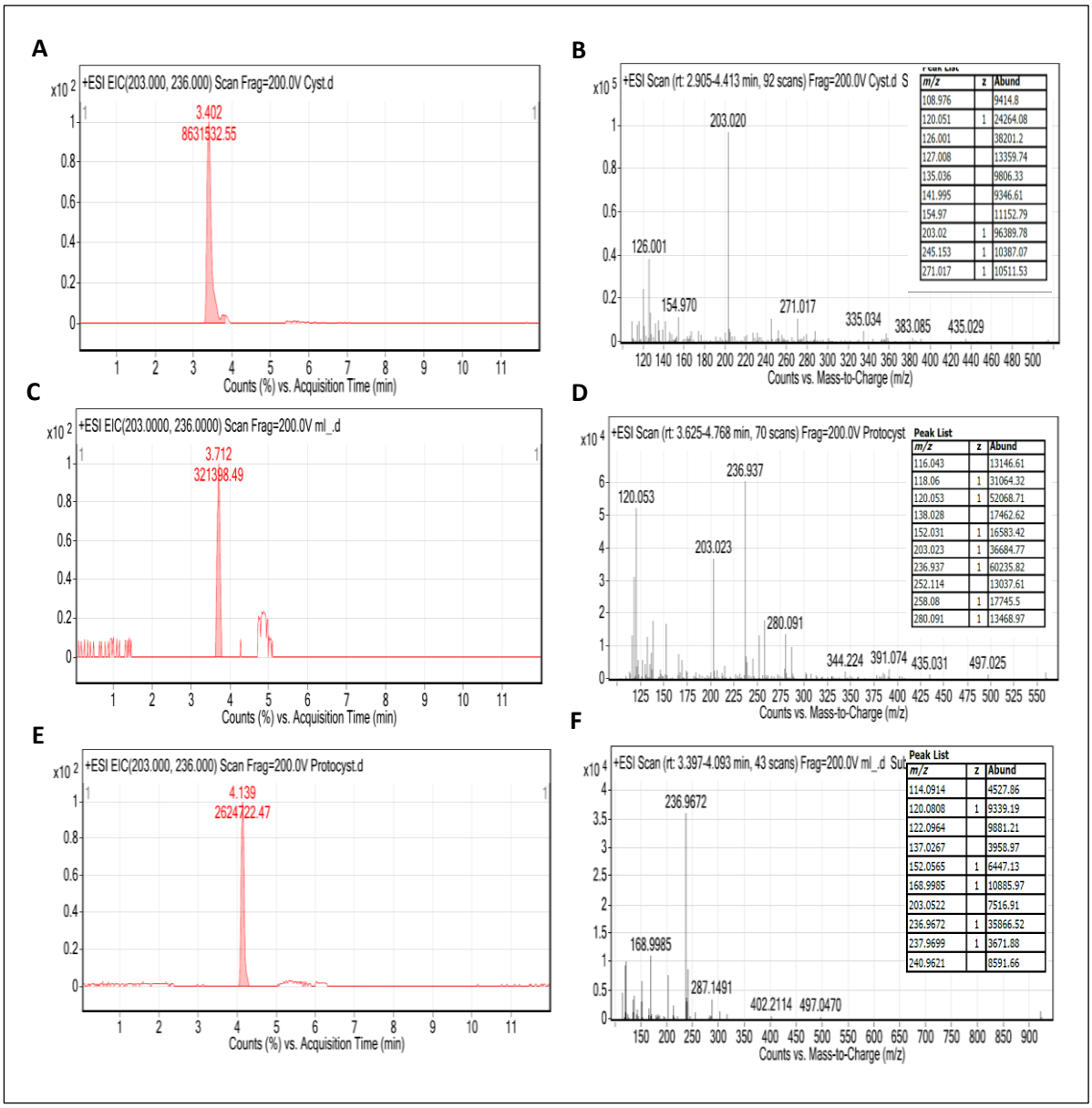

Figure 7-8: Separation of sugars: (A) Retention time for glucose formed from the cyst wall of *A. castallanii*, (B) Ion chromatogram peak for glucose extracted from the cyst wall of A. castallanii by hydrolysing in 1.1 M of aqueous H2SO4, (C) Retention time for glucose, (D) Ion chromatogram peak for glucose a which was obtained from the protocyst wall of A. castellanii by hydrolysing in 0.2 M of aqueous H2SO4. (E) Retention time for a methylated analogue of N-acetylglucosamine, (F) Ion chromatogram peak for possibly a methylated analogue of N-acetylglucosamine which was extracted from the protocyst wall of *A. castellanii* by hydrolysing in 0.2 M of aqueous H2SO4.

7.4 Discussion

This section provides an explanation for standard sugars, cellulose/chitin data that obtained from the LC/MS analysis and the comparison of findings related to cysts and protocysts from the LC/MS analysis and makes comparisons with previous studies for different microorganisms.

7.4.1 LC/MS analysis of sugars as standards

Liquid chromatography coupled with mass spectrometry (LC/MS) is a technique widely used to detect sugars in various applications. However, the separation of sugar through LC/MS is still complicated (Nagy *et al*., 2017). The method development and validation are always necessary for LC/MS and the MS detection limit is associated with the minimum efficiency of ionization related to the low acidic character of sugars (Licea-Perez *et al*., 2016). As the standard sugars are isomers, so the molecular weight for glucose, mannose and galactose is identical at 180.156 g/mol and for ribose and xylose it is also identical at 150.13 g/mol. In this study the sugars were analysed using LC/MS tool and were detected as their sodium ion adducts $(M+Na)^+$; this meant the ions detected were 23 m/z higher than the expected molar mass of the sugars (as a Na^+ weighs ~23 atomic mass units). The key step in the hydrolysis of cellulose and chitin polymers is breaking the 1,4 glycosidic bonds. The current study showed that a 1.1 M concentration of H_2SO_4 at a temperature of 140 °C for 2 hours gave optimal hydrolysis of cellulose into glucose monomers w. A concentration of 0.2 M of H_2SO_4, in the same conditions as used for cellulose, demonstrated a higher hydrolysis of chitin into *N*-acetyl-*d*-glucosamine monomers.

7.4.2 Comparing the data from the LC/MS analysis of cyst and protocyst walls with other microorganisms

The LC/MS analysis conducted in this study showed a glucose peak for treated cysts with a molecular ion peak of 203.020 m/z at a retention time of 3.402 min. A different retention time of 3.712 min was observed for protocysts, with fragment ion of 203.023 m/z which is matched with glucose peak of cyst in terms of molecular ion. However, another component was detected in the hydrolysed protocyst wall with a distinct molecular ion of 236.9672 m/z and a different retention time of 4.139 min, the molecular ion for this peak is protonated and not

sodiated (which meant the addition of a proton to the molecular ion of the detected component peak). These findings suggest that the walls of *Acanthamoeba* protocysts and cysts are different, with the protocyst wall containing glucose and possibly a methylated analogue of *N*-acetyl-*d*-glucosamine.

Previously, it has been reported that the 3-O-methyl-*N*-acetyl-*d*-glucosamine is a component of monosaccharides composition in *Clostridium thermocellum* bacteria, and this type of methylated sugar has a molecular ion of 235.2 m/z (Gerwig *et al*., 1989), which is comparable to that found in the current study as a methylated analogue of *N*-acetylglucosamine (i.e. m/z 236; detected as $[M+H]^+$). This is the first study to demonstrate the existence of potential component of *β*-methyl-*N*-acetyl-*d*-glucosamine in the wall of protocysts of *Acanthamoeba castellanii* (ATCC 50370). In the past, GC-MS was utilised to identify the composition of carbohydrates in the cyst walls of *Acanthamoeba castellanii* (ATCC 50492) and the findings showed that the walls of treated cysts contain mainly galactose (48.1%), glucose (44.4%) and a minor percentage (3.7%) of mannose and xylose (Dudley *et al*., 2009). The results of that research are in line with the data obtained from the LC/MS analysis in the present study, as the analysis of the hydrolysed cyst walls of *Acanthamoeba castellanii* (ATCC 50370)showed a glucose peak and the molecular ion was identical for both galactose and mannose (i.e. our data does not distinguish between these hexoses). Also, in the current study, both xylose and ribose peaks with the same molecular ions were detected by LC/MS in the cyst walls of *A. castellanii* (ATCC 50370), whereas Dudley *et al*. (2009) did not detect ribose within the cyst walls. There are two possible reasons for this difference: the first relates to the type of strain, as Dudley *et al*. (2009) used *A. castellanii* (ATCC 50492) and, in the current study, the strain analysed was *A. castellanii* (ATCC 50370). The second could be associated with the use of different instruments for the analysis, GC-MS in the earlier research and LC/MS in this study, as this could lead to cause limitation in the detection of the component.

In earlier research, Kliescikova *et al*. (2011) carried out analyses of different strains by utilized fluorescein labelled lectins to determine the carbohydrate components of the walls of *Acanthamoeba* cysts and protocysts. They demonstrated that the walls of exocysts contain the largest range of sugars,

including *α*-glucose/galactose, mannose, *α*-*N*-acetylglucosamine, galactose/*N*-acetylgalactosamine, galactosyl-*β*-*N*-acetylgalactosamine and *α*/*β*-*N*-acetylgalactosamine, whereas only a few sugars, including *α*-glucose/*α*-mannose, mannose and *N*-acetylglucosamine, were detected in endocysts (Kliescikova *et al*., 2011). However, the walls of protocysts reacted only with Concanavalin A lectin, and based on this observation, it has been suggested that the walls of protocysts contain only *α*-glucose and mannose (Kliescikova *et al*., 2011).

Conversely, in the present study, LC/MS analysis of the walls of protocysts obtained two different MS peaks for glucose and possibly *β*-methyl-*N*-acetyl-*d*-glucosamine, and these results contradict the findings reported in (Kliescikova *et al*., 2011). According to research by Dudley *et al*. (2009), the treated cyst walls of *A. castellanii* (ATCC 50492) contain cellulose, and the current study agrees with this observation. However, the findings from Kliescikova *et al*. (2011) showed that the cyst walls contain cellulose and *N*-acetylglucosamine and the protocyst wall consists of only cellulose. These findings are different from the results obtained in the present study. The possible explanation for this difference could be related to the strain types analysed, as Kliescikova *et al*. (2011) used three clinical isolates (V/01; B/04; N/05) which were taken from corneal scrapes of keratitis patients and another environmental strain (RB728/07SR), isolated from swimming pools, whereas in this study, *Acanthamoeba castellanii* (ATCC 50370) was analysed. Another potential reason may be due to the different methodologies employed: the present study utilised LC/MS analysis of hydrolysed cyst and protocyst walls of *A. castellanii* (ATCC 50370), which is superior to the fluorescein labelled lectins utilised by Kliescikova *et al*. (2011).

The auto-fluorescence spectroscopy was used to differentiate between *Acanthamoeba castellanii* and other pathogens, including *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Elizabethkingia miricola*, *Achromobacter ruhlandii* and *Candida albicans.* It has been shown that the cyst of *A. castellanii* is green under auto-fluorescence (Molyneux *et al*., 2015), so maybe Kliescikova *et al*. (2011) confused with auto-fluorescence and non-specific binding to the cyst walls of *Acanthamoeba.* On the other hand, It was found that the cyst walls of *Giardia* intestinalis filamentous are made up of a novel

β(1-3)-*N*-acetyl-D-galactopyranosamine homopolymer (Gerwig *et al*., 2002). Prior to that study, early work was carried out by Arroyo-Begovich *et al*. (1980), in which the walls of *Entamoeba invadens* cysts were purified and analysed, It was observed that the walls are composed mostly of chitin.

7.5 Future research work

Further analysis would be required to establish the exact structure of the postulated methyl-*N*-acetyl-*d*-glucosamine in order to provide a more detailed evaluation of the variations between mature cysts and other stage of *Acanthamoeba* protocysts. This would involve the use of nuclear magnetic resonance (NMR) spectroscopy to deduce the exact structure of the sample. This prospect will be very challenging as NMR is much less sensitive than LC/MS and both sample size and purity may be mitigating factors.

7.6 Conclusion

Overall, the LC/MS analysis showed a clear difference in the composition of monosaccharides between the walls of *Acanthamoeba* cysts and protocysts, as the cyst wall contains only one glucose peak which is indicative of cellulose, whereas the protocyst wall has two peaks, the glucose peak indicated cellulose and another peak that corresponds to a different component (possibly a methylated sugar of *N*-acetylglucosamine based upon MS data) which contradicts a prior study by Kliescikova *et al*. (2011). To this end, the current study indicates that the protocyst represents a distinctly separate stage of the lifecycle of *Acanthamoeba*.

Chapter Eight

General Conclusions

Chapter 8: General Conclusions

Acanthamoeba Keratitis (AK) is a serious, eye-threatening disease and infection caused by *Acanthamoeba* spp. it is often associated with wearing contact lenses. Misdiagnosis and ineffective treatment may lead to loss of vision. There are three different methods used to diagnosis of AK including: microbiological culture of corneal scrapings, *In vivo* confocal microscopy and quantitative polymerase chain reaction (qPCR) which well known for its sensitivity. As indicated in the previous chapters that the present medical treatment for AK entails biguanide either polyhexamethylene biguanide or chlorhexidine at concentration of 0.02% (v/v) as single drug or in combination with diamidines. Remarkably, in the current study, the octenidine hydrochloride had an excellent *in vitro* activity against cysts and trophozoites of both tested species of *Acanthamoeba* compared with PHMB and chlorhexidine.

This chapter summarises the work in this thesis aimed to optimizing the current treatment, developing novel compounds of amidoamines and new formulations of biguanides against cysts and trophozoites of *Acanthamoeba castellanii* (ATCC 50370) and *Acanthamoeba polyphaga* (ATCC 30461). To assess the toxicity on the human epithelial cell line in order to use the compounds which demonstrated superior *in vitro* activity and low toxicity for *in vivo* treatment. The other aims for my project are to test a range of agonists and antagonists on the cysts and protocysts formation to activate and block the receptor which involves in the encystment of *Acanthamoeba*. Also, to prevent the production of cysts and protocysts by utilizing cellulose synthesis inhibitors assays. Employing chemical analysis (LC/MS) to differentiate between the cyst and the potential new stage of *Acanthamoeba* named protocyst to confirm the presence of 3rd life cycle of *Acanthamoeba*.

A serious of anaesthetics and antibiotics have been tested in this study to investigate their possible *in vitro* effects against trophozoites and cysts of *Acanthamoeba* spp. As the anaesthetics applied *in vivo* to anesthetise the patient eye for a corneal scrape being performed and the antibiotics used prior to diagnosis of AK. Anaesthetics, in particular tetracaine, have shown superior antimicrobial activity against both cysts and trophozoites of *Acanthamoeba* spp.

and these drugs may perhaps lead to the limited sensitivity of culture observed in corneal scrape. The presence of benzalkonium chloride (BAC) in propamidine (Brolene®) eye drops and levofloxacin (Oftaquix®) as preserved appeared to have direct impact on the *in vitro* activity against trophozoites and cysts of both species. Different class of drugs have been tested in the current study including aspirin analogues, quaternary ammonium, antifungal, macrolide, antineoplastic for their antimicrobial activity and various compounds involving posaconazole, alexidine, didecyldimethylammonium chloride, miltefosine showed a higher *in vitro* activity against trophozoite and cyst and low toxicity toward the human epithelial cell line. The formulation of Lipodisq® carrier with biguanide compounds enhanced the antimicrobial activity from 1-fold to approximately 7-fold against trophozoites and cysts of *Acanthamoeba* spp.

Remarkably, the combination of chlorhexidine with Lipodisq® carrier showed a massive increased in the activity when it was tested over the time against Neff's cysts of *Acanthamoeba* as it demonstrated a 4.5 log reduction after 24 hours compared with the use of chlorhexidine alone which resulted in only 2 log kill at the same time point. The findings from encystment study indicated that the adrenoreceptor agonists bind to the receptor and had one effect and the antagonists interacted with the receptor in *Acanthamoeba* but gave an opposite impact and possibly these drugs bind to the evolution basic version of the receptor in *Acanthamoeba*. Based on the results from the encystment investigation, the β ultra-long agonist indacaterol at a lower concentration of 100 µM demonstrated a higher encystment level of 85.7%. This finding is very interesting as the indacaterol has an ability to bind to the receptor for a long duration and is considered to be the optimal ligand for the receptor in *Acanthamoeba*.

A significant finding was achieved from using the herbicide 2,6-dichlorobenzonitrile (DCB) which inhibits the biosynthesis of cellulose in *Acanthamoeba* by preventing the cellulose synthase enzyme. The higher concentration of 500 µM of BCB prevented the cysts formation at 17.7%. Best of our knowledge, no chemical investigation such as LC/MS, has been conducted in the past to analyse the cell wall components of the protocyst. Therefore, it is important to distinguish between the cyst stage and the potential form protocyst

of *Acanthamoeba*, as these findings enhance our understanding of resistant mechanism in *Acanthamoeba*. We have predicted that the protocyst wall made of *N*-acetylglucosamine which is present in the cyst wall of *Giardia intestinalis*. In this study, LC/MS analysis was performed for both cyst and protocyst walls alongside the standard sugars to determine the type of sugar. The LC/MS analysis data proposed that the cyst and protocyst of *Acanthamoeba* are different, as the analysis results of cyst wall indicated the existing of cellulose whereas the analysis of protocyst wall showed cellulose and a methylated analogue of *N*-acetyl-d-glucosamine.

The findings of this study have drawn a good attention for ophthalmologists to the use of adequate anaesthetics prior to corneal scarp and antibiotics for diagnosis of AK. The *in vitro* results of this study showed a massive inhibition of the viability of trophozoites and cysts by tetracaine, which means that this anaesthetic cannot be used *in vivo*. The current study has highlighted several new compounds that can be applied for clinical trial as an alternative *in vivo* treatment for AK. The alexidine, posaconazole, didecyldimethylammonium chloride and octenidine hydrochloride are currently not using in the treatment of AK, so it is possible to employing these drugs *in vivo* instead of using PHMB and chlorohexidine. The novel combination of biguanides in particular chlorhexidine incorporated with Lipodisq® carrier demonstrated excellent *in vitro* activity against trophozoites and the highly resistant cyst stage with minimum toxicity. The way forward is to use these combinations as an effective therapies for *in vivo* treatment against AK. For the first time this study has synthesised novel compounds of amidoamine involved myristoleyl-amidopropyl-dimethylamine (MOPD) and palmitoleyl-amidopropyl-dimethylamine (POPD). When these compounds were formulated into contact lens base solution at concentration of 0.0005% (w/v) and tested against trophozoites over the time, a complete kill at 4.5 log reduction after 24 hours was observed compared with only 3 log reduction for MAPD and PAPD as an existing compounds. This observation has raised the possibility to incorporate these compounds at a concentration of 0.0005% (w/v) into contact lens disinfectant solution as an alternative of MAPD disinfectant.

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9 References List

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